Supreme Court, U.S.
FILED
DEC 14 1979
MICHAEL RODAK, JR., CLERK

APPENDIX

In the Supreme Court of the United States

OCTOBER TERM, 1979

No. 79-136

LUTRELLE F. PARKER, ACTING COMMISSIONER OF PATENTS AND TRADEMARKS,

Petitioner

—v.—

MALCOLM E. BERGY, ET AL.

LUTRELLE F. PARKER, ACTING COMMISSIONER OF PATENTS AND TRADEMARKS,

Petitioner

—v.—

ANANDA M. CHAKRABARTY

ON WRIT OF CERTIORARI TO THE UNITED STATES COURT OF CUSTOMS AND PATENT APPEALS

PETITION FOR A WRIT OF CERTIORARI FILED JULY 27, 1979
CERTIORARI GRANTED OCTOBER 29 1979

In the Supreme Court of the United States

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INDEX

*Not reprinted in Joint Appendix. Citations are to appendix of Petition for Writ of Certiorari.

BOARD OF APPEALS

Serial No. 477,766

Docket No. 76-712

Filed August 16, 1976

IN THE MATTER OF THE APPLICATION OF
MALCOLM E. BERGY, JOHN H. COATS and
VEDPAL S. MALIK

DOCKET ENTRIES

DATE	PROCEEDINGS AND ORDERS

COUNSEL FOR APPELLANT: Roman Saliwanchik

COMMISSIONER OF PATENTS

August 30, 1976 Stipulation Under Rule 4.2(c) Correction or Modification of the Record, filed, and approved.

September 7, 1976 Above material received.

November 1, 1976 PRINTED TRANSCRIPT OF RECORD, FILED.

December 27, 1976 BRIEF FOR APPELLANT, filed.

February 8, 1977 Motion of Commissioner to extend time for filing brief to February 14, 1977, filed.

February 9, 1977 Above motion granted.

February 14, 1977 BRIEF FOR THE COMMISSIONER OF PATENTS AND TRADEMARKS, FILED.

February 16, 1977 Motion of appellants for an additional ten minutes for oral argument, filed.

February 18, 1977 Above motion granted.

March 3, 1977 Argued

October 6, 1977 Reversed, Rich, Judge. Concurring opinion by Judge Kashiwa. Dissenting opinion by Judge Miller with whom Judge Baldwin joins.

DATE	PROCEEDINGS AND ORDERS

October 27, 1977 PETITION FOR REHEARING BY COMMISSIONER, filed.

November 4, 1977 OPPOSITION TO PETITION FOR REHEARING BY APPELLANT, FILED.

November 23, 1977 Petition for rehearing denied. Judge Baldwin and Judge Miller would grant the petition.

November 30, 1977 FINAL MANDATE ISSUED TO THE COMMISSIONER OF PATENTS AND TRADEMARKS

February 14, 1978 Extension of time for Solicitor General to file certiorari in Supreme Court to April 22, 1978, granted by Justice Brennan.

April 20, 1978 Petition for Writ of Certiorari filed in Supreme Court of the United States, No. 77-1503.

July 26, 1978 Order of Supreme Court granting petition for writ of certiorari, vacating judgment, and remanding case for further consideration in light of *Parker* v. *Flook* (Appeal No. 77-512, decided 6/22/78).

August 8, 1978 Order restoring appeal to the calendar with supplementary briefs to be filed directed solely to the effect of *Parker* v. *Flook;* appellant's brief due on or before September 18; Commissioner's brief due on or before October 18; reply brief due on or before November 1. Case set for hearing November 6, 1978.

September 1, 1978 SUPPLEMENTAL BRIEF FOR APPELLANT, filed.

September 18, 1978 BRIEF FOR AMICUS CURIAE FOR UNIVERSITY OF CALIFORNIA, filed.

Sept. 18, 1978 Consent of appellant and Commissioner of Patents to University of Calif. filing brief Amicus Curiae, filed.

Sept. 18, 1978 Motion of American Patent Law Assn. for leave to file two typewritten Amicus Curiae briefs and extend time for filing requisite number of briefs to Sept. 22, 1978, filed.

DATE	PROCEEDINGS AND ORDERS

September 20, 1978 Above motion of APLA, granted.

Sept. 21, 1978 Consent by Commissioner to APLA brief Amicus curiae, filed.

September 22, 1978 BRIEF FOR AMICUS CURIAE FOR AMERICAN PATENT LAW ASSN., filed.

October 17, 1978 SUPPLEMENTAL BRIEF FOR THE COMMISSIONER OF PATENTS AND TRADEMARKS, FILED.

November 6, 1978 Argued.

March 29, 1979 Reversed, Rich, J. Concurring opinion by Judge Baldwin. Dissenting opinion by Judge Miller.

April 20, 1979 FINAL MANDATE ISSUED TO THE COMMISSIONER OF PATENTS AND TRADEMARKS

June 15, 1979 Letter from Supreme Court re extension of time for Commissioner to file Writ of Certiorari to July 27, 1979 in jacket.

July 27, 1979 Petition for writ of certiorari filed in the Supreme Court of the United States; No. 79-136.

October 29, 1979 Petition for Writ of Certiorari, granted.

Patent Appeal No. 76-712

APPLICATION OF MALCOLM E. BERGY, JOHN H. COATS AND VEDPAL S. MALIK, FILED JUNE 10, 1974, SERIAL NUMBER 477,766, FOR PROCESS

Abstract of the Disclosure

Microbiological process for preparing the antibiotic lincomycin at temperatures ranging from 18° C. to 45° C. using the newly discovered microorganism *Streptomyces vellosus*. The subject process advantageously results in the preparation of lincomycin without the concomitant production of lincomycin B (4′-depropyl-4′-ethyllincomycin). The absence of lincomycin B production results in increased lincomycin recovery efficiency.

Background of the Invention

The antibiotic lincomycin, formerly known as lincolnensin, can be produced by the microorganism *S. lincolnensis* var. *lincolnensis*, NRRL 2936, as disclosed in U.S. Patent 3,086,912. The incubation temperature range disclosed in said patent for the production of lincomycin is 18° to 40° C., and preferably 26° to 30° C. Also produced during the lincomycin fermentation is the compound known as lincomycin B. Though lincomycin and lincomycin B have activity against essentially the same spectrum of microorganisms, it is known that lincomycin B is significantly less active against said microorganisms than is lincomycin. Accordingly, lincomycin is the preferred antibiotic of the two.

In conducting the above fermentation, it is necessary to use a large amount of cooling water in most fermentation equipment to maintain the desired fermentation temperature. Further, the maintenance of a temperature within the range of 18° C. to 40° C., though essential for antibiotic production as disclosed above, is conducive to the development and proliferation of contaminating microorganisms in the fermentation vessel.

Brief Summary of the Invention

The subject invention concerns the fermentation preparation of lincomycin by the novel microorganism *Streptomyces vellosus* var. *vellosus*, NRRL 8037, at a temperature range of 18° to 45° C. It has been found, unexpectedly, that the titer of lincomycin produced at 45° C. is comparable to that which is produced at 28° C. The production of lincomycin at 28° C. and 45° C. for the microorganism of the subject invention is shown in the following table. The zone sizes of inhibition are given in millimeters. The test is a standard microbiological disc plate assay using 13 mm. paper discs.

Organism	*28° C.*	*45° C.*
Bacillus subtilis	21	18
Staphylococcus aureus	22	24
Sarcina lutea	31	29
Klebsiella pneumoniae	0	0
Escherichia coli	0	0
Salmonella schottmuelleri	0	0
Mycobacterium avium	22	25
Penicillium oxalicum	0	0

The results shown in the above table are unexpected since our tests have shown that *S. lincolnensis* var. *lincolnensis*, NRRL 2936, does not produce lincomycin when incubated at a temperature of about 45° C.

A distinct advantage in using this microorganism to prepare lincomycin is the need for less fermentor cooling capacity. The need for less cooling capacity is especially significant in high temperature climates and in areas having limited water supplies since water is the generally used means for cooling and maintaining fermentation temperatures. A further distinct advantage in the process of the subject invention is that lincomycin is produced without the concomitant production of lincomycin B.

Detailed Description of the Invention

The Microorganism

The novel actinomycete used according to this invention for the production of lincomycin is *Streptomyces*

vellosus. One of its strain characteristics is the production of lincomycin without the concomitant production of lincomycin B. Another of its strain characteristics is the production of comparable titers of lincomycin at a temperature of 28° C. and 45° C. A subculture of this living organism can be obtained upon request from the permanent collection of the Northern Regional Research Laboratories, Agricultural Research Services, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. Its accession number in this repository is NRRL 8037.

The microorganism of this invention was studied and characterized by Alma Dietz of the Upjohn Research Laboratory.

A thermoduric *Streptomyces* species isolated from Arizona soil produces the antibiotic lincomycin. The culture is readily differentiated from other lincomycin-producers as may be noted in Table 4. The thermoduric property, the microscopic characteristics of long, straight spore chains coiled at the tip, spores with long spines and hairs, and the distinctive antibiotic-producing capability of *Stretopmyces vellosus* are not reported for any of the *Streptomyces* species with blue-gray spore color mass cited in the significant *Streptomyces* taxonomy publications of Hütter [Hütter, R. 1967. Systematik der Streptomyceten unter besondere Berücksichtigung der von ihnen gebildeten Antibiotica. S. Karger, Basel], Krassilnikov [Krassilnivok, N. A. et *al.* 1966. Biology of Antibiotic-Producing Actinomycetes, Akademiya Nauk SSSR. Edited by Ya. I. Rautenstein. Published for the U.S. Department of Agriculture and the National Science Foundation, Washington, D.C. by the Israel Program for Scientific Translations], Kutzner [Kutzner, H. J. 1956. Beitrag zur Systematik and Ökologie der Gattung *Streptomyces* Waksm. et Henrici. Diss. Landw. Hochst. Hohenhein], Pridham, et al [Pridham, T. G., C. W. Hesseltine, and R. G. Benedict. 1958. A guide for the classification of streptomycetes according to selected groups. Placement of strains in morphological sections. Applied Microbiol. *6*:52-79], Shirling and Gottlieb [Shirling, E. B., and D. Gottlieb. 1968. Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study.

Int. J. of Syst. Bacteriol. *18*:69-189; Shirling, E. B. and D. Gottlieb. 1968. Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. of Syst. Bacteriol. *18*:279-392; Shirling, E. B. and D. Gottlieb. 1969. Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. of Syst. Bacteriol. *19*:391-512; and Shirling, E. B. and D. Gottlieb. 1972. Cooperative description of type strains of *Streptomyces* V. Additional descriptions. Int. J. of Syst. Bacteriol. *22*:265-394], Trejo [Trejo, W. H. and R. E. Bennett. 1963. Streptomyces species comprising the blue-spore series. J. Bacteriol. *85*:676-690], or Waksman [Waksman, S. A. 1961. The actinomycetes, vol. 2, Classification, identification, and descriptions of genera and species. The Williams & Wilkins Co., Baltimore]. Therefore, it is proposed that this isolate be designated *Streptomyces vellosus* Dietz, sp.n. and that this type species be designated the type variety *Streptomyces vellosus* var. *vellosus*. The species and variety designations are made in accordance with the Rules set forth in the International Code of Nomenclature of Bacteria [International Code of Nomenclature of Bacteria. 1966. Edited by the Editorial Board of the Judicial Commission of the International Committee on Nomenclature of Bacteria. Int. J. Syst. Bacteriol. *16*:459-490].

Streptomyces vellosus Dietz, sp. n.

Color characteristics. Aerial growth blue-gray to gray. Melanin-positive. Color on Ektachrome [Dietz, A. 1954. Ektachrome transparencies as aids in actinomycete classification. Ann. N.Y. Acad. Sci. *60*:152-154] is given in Table 1. Reference color characteristics are given in Table 2. *Streptomyces vellosus* may be placed in the Blue (B) and White (W) color series of Tresner and Backus [Tresner, H. D., and E. J. Backus. 1962. System of color wheels for Streptomycete taxonomy. Applied Microbiol. *11*:335-338].

Microscopic characteristics. Spore chains long, straight with a tight to open coil at the tip. Spore chains spiral

(S) as defined by Pridham *et al.* [Pridham, T. G., C. W. Hesseltine, and R. G. Benedict. 1958. A guide for the classification of streptomycetes according to selected groups. Placement of strains in morphological sections. Applied Microbiol. *6*:52-79]. Spores large, mostly oval. Spore surface adorned with long spines and hairs. Spore surface hairy as defined by Dietz and Mathews [Dietz, A. and J. Mathews. 1971. Classification of *Streptomyces* spore surfaces into five groups. Appl. Microbiol. 21:527-533].

Cultural and biochemical characteristics. See Table 3.

Carbon utilization. The growth of *S. vellosus* on carbon compounds was determined using the synthetic media of Pridham and Gottlieb [Pridham, T. G., and D. Gottlieb, 1948. The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. *56*:107-114] and of Shirling and Gottlieb [Shirling, E. B., and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. Int. J. of Syst. Bacteriol. *16*:313-330]. In the former, the culture showed trace growth on the control (basal medium without a carbon compound), dulcitol, *D*-sorbitol, sodium oxalate, and sodium tartrate, moderate growth on sodium acetate, sodium citrate, and sodium succinate; good growth on *D*-xylose, *L*-arabinose, rhamnose, *D*-fructose, *D*-galactose, *D*-glucose, *D*-mannose, maltose, sucrose, lactose, celloboise, raffinose, dextrin, inulin, soluble starch, glycerol, *D*-mannitol, and inositol. The culture did not grow on salicin, phenol, cresol, sodium formate or sodium salicylate. In the medium of Shirling and Gottlieb the culture grew slightly on the negative control (basal medium without a carbon compound) as well on the positive control (basal medium with *D*-glucose). Growth was equal to or better than on the basal medium plus glucose on *D*-xylose, inositol, *D*-mannitol, rhamnose and raffinose. Growth was significantly better than on the negative control but less than on the *D*-glucose control on *L*-arabinose, sucrose, and *D*-fructose. Growth on cellulose was doubtful.

Temparture. S. vellosus is a thermoduric actinomycete. It grows well at temperatures of 18-55 C. Optimum growth occurs at 28-37 C. in 10-14 days; at 45 C. in 48 hours.

Antibiotic-producing properties. S. vellosus produces the antibiotic lincomycin.

Source. Soil from Arizona.

Type culture. Streptomyces vellosus Dietz, sp.n NRRL 8037.

Type variety. Streptomyces vellosus var. *vellosus* NRRL 8037.

TABLE 1

*Appearance of Streptomyces vellosus on Ektachrome**

Agar Media	Surface	Reverse
Bennett's	Gray	Tan-brown
Czapek's sucrose	Trace gray	Yellow-tan
Maltose tryptone	——	Brown
Peptone-iron	——	Brown
0.1% Tyrosine	Trace blue-gray	Brown
Casein-starch	Blue-gray	Tan-brown

* Dietz, A. 1954. Ektachrome transparencies as aids in actinomycete classification. Ann. N.Y. Acad. Sci. *60*:152-154.

TABLE 2

Reference Color Characteristics of Streptomyces vellosus

Agar medium	Determination	Color Harmony Manual 3rd ed., 1948 •	NBS Circular 553, 1955 ••
		(g)	
Bennett's	S	15ba to 5ba blue tint to shell pink	184m very pale blue 189gm bluish white 9m pinkish white
	R	3gc light tan	76gm light yellowish brown
	P	3ie camel, maple sugar, Tan	76m light yellowish brown 77g moderate yellowish brown
Czapek's sucrose	S	3cb sand	—
	R	3ec bisque, light beige	79gm light grayish yellowish brown 90 grayish yellow
	P	—	—
Maltose-tryptone	S	5ba shell pink	9 pinkish white
	R	31g adobe brown, cinnamon brown, light brown	77gm moderate yellowish brown
	P	3ie camel, maple sugar, tan	76m light yellowish brown 77g moderate yellowish brown

TABLE 2 CON'T

Reference Color Characteristics of Streptomyces vellosus

Agar medium	Determination	Color Harmony Manual 3rd ed., 1948 •	NBS Circular 553, 1955 ••
Hickey-Tresner	S	15ba to 3cb blue tint to sand	184m very pale blue 189m bluish white
	R	31g adobe brown, cinnamon brown, light brown	77m moderate yellowish brown
	P	31e cinnamon, yellow maple	76m light yellowish brown
Yeast-extract-malt extract (ISP-2)	S	15ba to 2ba blue tint to pearl, shell tint	184m very pale blue 189gm bluish white 92gm yellowish white
	R	3b	74g strong yellowish brown 76m light yellowish brown
	P	31e	74g strong yellowish brown 76m light yellowish brown
Oatmeal (ISP-3)	S	15cb cloud blue	184m very pale blue 190g light bluish gray
	R	2gc bamboo, chamois	90 gm grayish yellow
	P	—	—
Inorganic-salts starch (ISP-4)	S	19dc aqua gray	149g pale green 190m light bluish gray

TABLE 2 CON'T

Reference Color Characteristics of Streptomyces vellosus

Agar medium	Determination	Color Harmony Manual 3rd ed., 1948 •	NBS Circular 553, 1955 ••
	R	2fb bamboo, buff, straw, wheat	87g moderate yellow 89m pale yellow
	P	—	—
Glycerol-asparagine (ISP-5)	S	15ba blue tint	184m very pale blue 189gm bluish white
	R	2fb bamboo, buff, straw, wheat	87g moderate yellow 89m pale yellow
	P	—	—

S = Surface
R = Reverse
P = Pigment

(g) = all from glossy surface of color chip
g = glossy surface of color chip
m = matte surface of color chip
gm = glossy or matte suface of color chip

• Jacobson, E., W. C. Granville, and C. E. Foss. 1948. Color harmony manual, 3rd ed. Container Corporation of America, Chicago, Illinois.

•• Kelly, K. L., and D. B. Judd. 1955. The ISCC-NBS method of designating colors and a dictionary of color names. U.S. Dept. Comm. Circ. 553.

TABLE 3

Cultural and Biochemical Characteristics of Streptomyces vellosus

Medium	Surface (aerial growth)	Reverse	Other Characteristics
Agar media			
Peptone-iron	None at 28 C. Gray at 45 C.	Brown	Brown pigment Melanin-positive
Calcium-malate	Trace white	Colorless	No pigment Malate not solubilized
Glucose-asparagine	Pale pink-white	Cream at 28 C. Olive at 45 C.	Yellow pigment at 28 C. No pigment at 45 C.
Skim milk	Trace gray at 28 C. None at 45 C.	Tan brown	Tan brown pigment Casein not solubilized
Tyrosine	Trace gray at 28 C. Fair gray at 45 C.	Brown at 28 C. Tan at 45 C.	Brown pigment at 28 C. Tan pigment at 45 C. Tyrosine not solubilized at 28 C. Tyrosine solubilized under growth at 45 C.
Xanthine	None at 28 C. Pink white at 45 C.	Yellow	Yellow pigment Xanthine not solubilized

TABLE 3 CON'T

Cultural and Biochemical Characteristics of Streptomyces vellosus

Medium	Surface (aerial growth)	Reverse	Other Characteristics
Nutrient starch	None at 28 C. Pink-white at 45 C.	Yellow tan at 28 C. Yellow at 45 C.	Yellow tan pigment at 28 C. Yellow pigment at 45 C. Starch not hydrolyzed
Yeast extract-malt extract	Pink white (best at 45 C.)	Red tan at 28 C. Tan at 45 C.	Red tan pigment at 28 C. Tan pigment at 45 C.
Bennett's	Pale cottony blue-white	Tan	Tan pigment
Czapek's sucrose	Pale cream pink	Yellow	Yellow pigment
Maltose-tryptone	Pale cottony blue-white	Brown	Brown pigment
Hickey-Tresner	Pale cottony blue-white	Orange-tan	Pale tan pigment
Peptone-yeast extract-iron (ISP-6)	None	Brown	Brown pigment Melanin-positive
Tyrosine (ISP-7)	Pink-white	Brown	Brown pigment Melanin-positive

TABLE 3 CON'T

Cultural and Biochemical Characteristics of Streptomyces vellosus

Medium	Surface (aerial growth)	Reverse	Other Characteristics
Gelatin Media			
Plain	—	—	Brown pigment at surface Olive pigment top half No liquefaction
Nutrient	—	—	Brown pigment at surface Tan pigment throughout No liquefaction—2 tubes Trace liquefaction—2 tubes
Broth media			
Synthetic nitrate	—	—	Colorless vegetative growth throughout broth and at base No pigment Nitrate not reduced to nitrite
Nutrient nitrate	—	—	Colorless compact bottom growth Yellow pigment Nitrate not reduced to nitrite
Litmus milk	White-gray aerial growth on surface ring	—	Brown pigment Litmus reduced pH 6.8

TABLE 4

Comparison of Streptomyces vellosus with other lincomycin-producers

	S. vellosus NRRL 8037	*S. lincolnensis* NRRL 2936	*S. espinosus* NRRL 3890
Aerial mycelium	Blue-Gray to gray	Cream to pink to gray	Gray green
Melanin	Positive	Positive	Negative
Spore chains	Spiral (S)-very long and coiled at tip	Long flexuous (RF)	Short, straight to flexuous to open spiral (RF, RA)—short
Spores	Spherical	Rectangular	Spherical
Spore surface	Long spines and hairs	Smooth with surface detail	Thorny to spiny—transition to hairy on some spines
Calcium malate agar	Malate not solubilized	Malate not solubilized	Malate not solubilized
Skim milk agar	Casein not solubilized	Casein not solubilized	Casein solubilized
Tyrosine	Not solubilized	Solubilized	Solubilized
Xanthine	Not solubilized	Solubilized around growth	Not solubilized
Nutrient starch	Starch not hydrolyzed	Starch hydrolyzed	Starch hydrolyzed

TABLE 4 CON'T

Comparison of Streptomyces vellosus with other lincomycin-producers

	S. pseudogriseolus chemovar linmyceticus NRRL 3985	*S. variabilis chemovar liniabilis* NRRL 5618
Aerial mycelium	Gray to white to red	Gray to white
Melanin	Negative	Negative
Spore chains	Short to moderately long straight (RF) to open spiral (RA) to spiral (S)	Short to moderately long flexuous (RF) to open spiral (RA)
Spores	Oval to oblong	Oval to oblong
Spore surface	Sparsely spiny to smooth	Smooth to poorly warty to spiny
Calcium malate agar	Malate not solubilized	Malate solubilized
Skim milk agar	Casein solubilized under growth	Casein solubilized
Tyrosine	Solubilized	Solubilized
Xanthine	Solubilized	Solubilized
Nutrient starch	Starch hydrolyzed	Starch hydrolyzed around growth

Lincomycin is produced by the novel microorganism of the subject invention when said microorganism is grown in an aqueous nutrient medium under submerged aerobic conditions. It is to be understood also that for the preparation of limited amounts surface cultures and bottles can be employed. The organism is grown in a nutrient medium containing a carbon source, for example, an assimilable carbohydrate, and a nitrogen source, for example, an assimilable nitrogen compound or proteinaceous material. Preferred carbon sources include glucose, brown sugar, sucrose, glycerol, starch, cornstarch, lactose, dextrin, molasses, and the like. Preferred nitrogen sources include corn steep liquor, yeast, autolyzed brewer's yeast with milk solids, soybean meal, cottonseed meal, cornmeal, milk solids, pancreatic digest of casein, distillers' solids, animal peptone liquors, fishmeal, meat and bone scraps, and the like. Combinations of these carbon and nitrogen sources can be used advantageously. Trace metals, for example, zinc, magnesium, manganese, cobalt, and the like, usually need not be added to the fermentation media since tap water and unpurified ingredients are used as media components.

Production of lincomycin by the process of the subject invention can be effected at a temperature of about 18° to about 45° C., and preferably at a temperature of about 20° C. to about 45° C. Ordinarily, optimum production of lincomycin is obtained in about two to ten days. The final pH is dependent, in part, on the buffers present, if any, and in part on the initial pH of the culture medium.

When growth is carried out in large vessels and tanks, it is preferable to use the vegetative form, rather than the spore form, of the microorganism for inoculation to avoid a pronounced lag in the production of lincomycin and the attendant inefficient utilization of the equipment. Accordingly, it is desirable to produce a vegetative inoculum in a nutrient broth culture by inoculating this broth culture with an aliquot from a soil or a slant culture. When a young, active vegetative inoculum has thus been secured, it is transferred aseptically to large vessels or tanks. The medium in which the vegetative inoculum is produced can be the same as, or different from, that

utilized for the production of lincomycin, as long as it is such that a good growth of the microorganism is obtained.

The lincomycin produced by the subject process can be recovered by the procedure disclosed in U.S. 3,086,912.

In preferred recovery process, lincomycin is recovered from its culture medium by separation of the mycelia and undissolved solids by conventional means, such as by filtration and centrifugation. Lincomycin is then recovered from the filtered or centrifuged broth by passing said broth over a resin which comprises a non-ionic macro porous copolymer of styrene crosslinked with divinylbenzene. Resins of this type are disclosed in U.S. Patent 3,515,717. Exemplary of this type of resin is Amberlite XAD-2. Lincomycin is eluted from the resin with a solvent system consisting of methanol-water (95:5 v/v). Bioactive eluate fractions are determined by a standard microbiological disc plate assay using the microorganism *Sarcina lutea*. Biologically active fractions are combined, concentrated to an aqueous solution which is then freeze dried. The freeze dried material is then triturated with methylene chloride. The methylene chloride extract is concentrated to dryness and the residue triturated with acetone. The filtrate is mixed with ether to give a precipitate which is separated. The remaining filtrate is mixed with methanolic hydrogen chloride (1 N) to precipitate colorless lincomycin hydrochloride. This precipitate is isolated by filtration and crystallized from water-acetone to give crystalline lincomycin hydrochloride.

The process of the subject invention is not limited to the particular microorganism fully described by the cultural characteristics disclosed herein. It is intended that this invention also include other lincomycin-producing strains or mutants of the said microorganism which can be produced by procedures well known in the art, for example, by subjecting the novel microorganism to x-ray or ultraviolet radiation, nitrogen mustard, phage exposure, and the like.

Hereinafter is described a non-limiting example of the process of the present invention. All percentages are by weight and all solvent portion mixtures are by volume unless otherwise noted.

EXAMPLE 1

Part A. FERMENTATION AT 28° C.

A soil slant of *Streptomyces vellosus*, NRRL 8037, is used to inoculate a series of 500-ml. Erlenmeyer flasks containing 100-ml. of sterile seed medium consisting of the following ingredients:

Glucose monohydrate	25 g./liter
Pharmamedia *	25 g./liter
Tap water q.s.	Balance

Presterilization pH=7.2

* Pharmamedia is an industrial grade of cottonseed flour produced by Traders Oil Mill Company, Fort Worth, Texas.

The flasks are grown for 3 days at 28° C., on a rotary shaker.

Seed inoculum, described above, is used to inoculate a series of 500-ml. Erlenmeyer fermentation flasks containing 100-ml. of sterile medium consisting of the following ingredients:

Glucose monohydrate	15 g./liter
Wilson's Peptone Liquor No. 159 *	15 g./liter
Difco Yeast Extract **	2.5 g./liter
Tap water q.s.	Balance

Presterilization pH=6.0

* Wilson's Peptone Liquor No. 159 is a preparation of hydrolyzed proteins of animal origin.

** Supplied by Difco Laboratories, Detroit, Michigan.

The flasks are inoculated with 5 ml. of seed inoculum per 100 ml. of fermentation medium. The flasks are then incubated at 28° C. on a rotary shaker operating at 250 rpm with a 6 cm. stroke. The flasks are harvested after 96 hours of fermentation.

Part B. FERMENTATION at 45° C.

Seed inoculum, as described above in Part A, is used to inoculate a series of 500-ml. Erlenmeyer fermentation flasks containing 100 ml. of sterile medium consisting of the following ingredients:

Glycerol	30 g./liter
NZ-amine B *	20 g./liter
Difco Yeast Extract	2 g./liter
Sodium chloride	3 g./liter
Tap water q.s.	Balance

Presterilization pH=7.2

* A bulk peptone in powder form obtained by the pancreatic digestion of casein.

The flasks are inoculated with 5 ml. of seed inoculum per 100 ml. of fermentation medium. The flasks are then incubated at 45° C. on a rotary shaker operating at 250 rpm with a 6 cm. stroke. The flasks are harvested after 96 hours of fermentation.

Part C. RECOVERY

The lincomycin produced in the fermentations as disclosed in Parts A and B is recovered in pure form by first filtering the fermentation beers using diatomaceous earth and filter aid. The filter cake is washed with water and the wash is combined with the clear filtrate. The clear filtratewash is then passed over a column containing Amberlite XAD-2 resin packed in water. The lincomycin is eluted from the resin with methanol-water (95:5 v/v). Fractions are collected and analyzed by thin layer chromatography on silica gel G using the solvent system consisting of methyl ethyl ketone-acetone-water (186:52:20 v/v). Active fractions are combined and concentrated to an aqueous and freeze dried. The dry material is then triturated with methylene chloride. The methylene chloride extract is concentrated to dryness. The resulting residue is triturated with acetone.

Insoluble material is removed by filtration and the remaining filtrate is mixed with ether. Again, precipitated material is removed by filtration and the remaining filtrate is mixed with methanolic hydrogen chloride (1 N). The resulting precipitated colorless lincomycin hydrochloride is isolated by filtration. This material is converted to the crystalline form by crystalization from water-acetone.

The amount of lincomycin B is a normal fermentation of *Streptomyces lincolnensis* var. *lincolnensis* will vary with the media composition, incubation time and temperature, aeration, etc. Under normal operating conditions amounts of lincomycin B in such a fermentation will range from 5 to 10% of the total lincomycin present. The lincomycin B is removed by repeated recrystallization of the lincomycin product in suitable solvents, for example, water-acetone mixtures, or water-lower alcohol mixtures. Since the process of the subject invention does not produce lincomycin B, these crystallizations are unnecessary.

CLAIMS

—1—

A novel process for preparing the antibiotic lincomycin which comprises cultivating *Streptomyces vellosus*, having the identifying characteristics of NRRL 8037, and lincomycin-producing mutants thereof, in an aqueous nutrient medium under aerobic conditions until substantial antibiotic activity is imparted to said medium by the production of lincomycin.

—2—

A process, according to claim 1, wherein the cultivation is conducted at a temperature range of about 18° C. to about 45° C.

—3—

A process, according to claim 1, wherein said aqueous nutrient medium contains a source of assimilable carbohydrate and assimilable nitrogen.

—4—

A process, acording to claim 1, wherein said lincomycin is isolated from the fermentation broth.

OATH AND POWER OF ATTORNEY

Being duly sworn, We, Malcolm E. Bergy and John H. Coates and Vedpal S. Malik depose and say:

that we are citizens of U.S.A., U.S.A., and Canada, respectively residing in the City of Kalamazoo, County of Kalamazoo, State of Michigan; in the City of Kalamazoo, County of Kalamazoo, State of Michigan; in the City of Kalamazoo, County of Kalamazoo, State of Michigan, respectively,

that we have read the foregoing specification and claims and we verily believe ourselves to be the original first, and joint inventors of the invention or discovery in PROCESS described and claimed therein;

that we do not know and do not believe that this invention was ever known or used before our invention or discovery thereof, or patented or described in any printed publication in any country before our invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than twelve months before this application; and

that this invention or discovery has not been patented in any country foreign to the United States on an application filed by us or our legal representatives or assigns more than twelve months before this application; and

that no application for patent on this invention or discovery has been filed by us or our legal representatives or assigns in any country foreign to the United States, except as follows: None.

And we hereby appoint Roman Saliwanchik and John Kekich (Registration Nos. 21,023 and 17,002, respectively), c/o Patent Law Department, The Upjohn Company, Kalamazoo, Michigan 49001 our attorney(s) or agent(s) with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith.

Inventor: /s/ MALCOLM E. BERGY
Malcolm E. Bergy

Post Office Address: 2617 Lomond Drive, Kalamazoo, Michigan 49008

Inventor: /s/ JOHN S. COATS
John H. Coats

Post Office Address: 3419 Old Colony Road, Kalamazoo, Michigan

Inventor: /s/ VEDPAL S. MALIK
Vedpal S. Malik

Post Office Address: 821 West Lovell, Kalamazoo, Michigan

STATE OF MICHIGAN
COUNTY OF KALAMAZOO, ss.

On this 6th day of June, 1974, personally appeared before me the above-named Malcolm E. Bergy and John H. Coats and Vedpal S. Malik, the persons described in the above application for patent, who signed the foregoing in my presence, and made oath before me to the allegations set forth therein as being under oath, on the day and year aforesaid.

/s/ KENNETH L. MCCLISH
Notary Public

SEAL

My Commission Expires: March 28, 1976.

This form may be executed only when attached to a complete application as the last page thereof.

Approved Single Signature Form
Joint Inventors
(No. 1585)

PRELIMINARY AMENDMENT, JANUARY 17, 1975

January 14, 1975

To the Commissioner
Washington, D.C. 20231

Sir:

Please amend the above-identified application as follows:

In the Claims:

Please add the following claim 5:

5. A biologically pure culture of the microorganism *Streptomyces vellosus*, having the identifying characteristics of NRRL 8037, said culture being capable of producing the antibiotic lincomycin in a recoverable quantity upon fermentation in an aqueous nutrient medium containing assimilable sources of carbon, nitrogen and inorganic substances.

REMARKS

Claims 1-4 are in the case. Claim 5 is being added by this amendment. Basis for claim 5 can be found throughout the disclosure. The identity of the microorganism is given in the detail description beginning on page 4 and extending through page 16.

Respectfully submitted,

/s/ ROMAN SALIWANCHIK
Roman Saliwanchik
Attorney

AMENDMENT, JANUARY 27, 1975

January 23, 1975

To the Commissioner of Patents
Washington, D.C. 20231

Sir:

In response to the Office Action dated January 20, 1975, please amend the above-identified application as follows:

In the Specification:

Page 1. Please delete the present title and insert—

PROCESS FOR PREPARING LINCOMYCIN—.

REMARKS

Claims 1-4 have been examined by the Examiner. Claim 5 was added by an amendment dated January 14, 1975, which, apparently, the Examiner had not received prior to the subject action.

The applicants are submitting, as a part of this response, an affidavit by the attorney for the applicants who attests that restrictions on availability of the microorganism will be removed upon the granting of a patent. Further, it is attested that the culture will be maintained by the repository for the effective life of the patent. In view of this affidavit, it is respectfully requested that the Rejection under 35 USC 112 be withdrawn.

In light of the above and the amendment submitted on January 14, 1975, with the addition of claim 5, it is respectfully requested that claims 1-5 be allowed.

Respectfully submitted,

/s/ ROMAN SALIWANCNIK
Roman Saliwanchik
Attorney

AFFIDAVIT OF SALIWANCHIK

STATE OF MICHIGAN

COUNTY OF KALAMAZOO, ss.

ROMAN SALIWANCHIK, being duly sworn, deposes and says:

THAT, as attorney for the applicants, he hereby avers:

1. that all restrictions on the availability of the culture deposits disclosed in the subject patent application will be irrevocably removed on the granting of a patent for the subject application, and
2. that the cultures will be maintained in a viable state throughout the effective life of the patent, and
3. that should the repository notify the applicants that one or more of the cultures is non-viable the applicants will replace the non-viable culture with a viable culture in the repository to be maintained for the effective life of the patent which discloses the same.

FURTHER, deponent sayeth not.

/s/ ROMAN SALIWANCHIK
Roman Saliwanchik

* * * *

AFFIDAVIT OF GRADY

February 18, 1975

STATE OF MICHIGAN

COUNTY OF KALAMAZOO, ss.

JOSEPH E. GRADY, being duly sworn, deposes and says:

THAT, in 1948 I received a B.S. degree from the University of Scranton in Pennsylvania; that, in 1951 I received an M.S. degree in Bacteriology from Purdue University; that, in 1958 I received a Ph.D. degree in Bacteriology from Purdue University;

THAT, since 1958 I have been in the employ of The Upjohn Company as a microbiologist; that, my major responsibility with The Upjohn Company is in the antibiotic field; that, I have actively engaged in a program of antibiotic screening and culture development; that, I presently supervise a program of antibiotic screening and culture development; that, in this program I actively supervise the screening of microorganisms in an attempt to find new antibiotics;

THAT, I read and studied application Serial No. 477,766; and being thus qualified, further deposes and says:

The "biologically pure culture" of Claim 5 is a well-defined product of a microbioligist which is capable of producing the desired antibiotic lincomycin under controlled fermentation conditions. In contrast, the soil source in which the microorganism was discovered is a complex microbial environment which, as such, could not be used to produce a desired product under any known fermentation conditions. The microbial complexity of soils was reviewed by Dr. S. A. Waksman in his book, *The Actinomycetes Vol. 1*, The Williams & Wilkins Company, 1959, where at page 31 he states:

> "At the surface of certain soils, actinomycetes, as measured by the number of colonies produced on agar plates, made up 9 to 15 percent of the total population, or a total of 743,000 to 933,000 per gram of soil. At a depth of 30 inches, the numbers dropped to 240,000 per gram, but the percentage rose to about 66. In California soils, the numbers varied from 380,000 to 1,890,000 per gram, and the percentage of the total population from 19 to 45."

Thus, actinomycetes, which is the group of microorganisms to which the microorganism of application Serial No. 477,766 belongs, make up a significant portion of the microbial flora of soils. Further, the actinomycetes in soils themselves are complex in kind and metabolic activities. Along with the actinomycetes, soil is host for numerous other microbes, among which are bacteria and fungi.

In summary, soil contains a complex jungle of microorganisms. It is only by the discovery and skills of the microbiologist that biologically pure cultures of microorganisms come into existence. Thus, the biologically pure culture" of Claim 5 is *not* found in nature.

FURTHER deponent sayeth not.

/s/ JOSEPH E. GRADY
Dr. Joseph E. Grady

* * * *

AFFIDAVIT OF DIETZ

February 18, 1975

STATE OF MICHIGAN

COUNTY OF KALAMAZOO, ss.

ALMA DIETZ, being duly sworn, deposes and says:

THAT, I received a Bachelor of Arts degree from American International College, Springfield, Massachusetts, in 1944; that from 1944-1946 I was a lab instructor in biology at the American International College; that during the summer of 1945 I took a phycology course given at the Marine Biological Lab, Woods Hole, Massachusetts; that in the summer of 1946 I assisted in the phycology course at Marine Biological Lab; that from 1946-1948 I did graduate work in botany at the University of Michigan; that from 1948 to the present time I have been in the employ of The Upjohn Company; that my primary responsibilities with The Upjohn Company concern the maintenance and identification of actinomycete cultures; that during the course of this work I routinely study actinomycetes isolated from various sources, such as soil, in an attempt to determine whether a new species of microorganism has been discovered; that I am the author or co-author of numerous publications concerning the taxonomic features of microorganisms, particularly the streptomyces; that I studied and characterized the biologically pure culture disclosed in application Serial No. 477,766; and being thus qualified, further deposes and says;

The taxonomic description of *Streptomyces vellosus* given in application Serial No. 477,766 was conducted on a biologically pure culture of *Streptomyces vellosus*. An impure culture of *S. vellosus* would give taxonomic results different from those given in application Serial No. 477,766. The different results would depend on the nature of the biological impurity, i.e., if it was a bacterium, another Streptomycete or a fungus. Thus, a meaningful taxonomic description, such as is given in application

Serial No. 477,766 must be done on a biologically pure culture of the microorganism.

Microorganisms found in the soil are complex in kind and cannot be taxonomically characterized without first producing a biologically pure culture. This clearly establishes that the "biologically pure culture" of Claim 5 is *not* found in nature; it is the product of a microbiologist.

FURTHER deponent sayeth not

/s/ ALMA DIETZ
Alma Dietz

* * * * *

AFFIDAVIT OF MILLER

February 18, 1975

STATE OF MICHIGAN

COUNTY OF KALAMAZOO, ss.

THOMAS L. MILLER, being duly sworn, deposes and says:

THAT, in 1961 I received an A.B. degree from Indiana State University; that, in 1963 I received a M.S. degree from the University of Wisconsin; that, in 1966 I received a Ph.D. degree from the University of Wisconsin majoring in biochemistry and minoring in bacteriology;

THAT, since 1966 I have been in the employ of The Upjohn Company initially as a Research Associate, then as a Section Head, Research Section Head, and since 1970 I have been Research Manager of Fermentation Research and Development;

THAT, I presently supervise microbiologists in the various aspects of fermentation research and development, with a large part of the work directly related to antibiotics;

THAT, I read and studied application Serial No. 477,766; and being thus qualified, further deposes and says:

The fermentation disclosed in application Serial No. 477,766 is conducted with a biologically pure culture of *S. vellosus*. A biologically *impure* culture of *S. vellosus* would *not* give the desired fermentation product under the conditions disclosed in application Serial No. 477,766, or possibly under *any* fermentation conditions. For example, if the biological impurity in a biologically *impure* culture of *S. vellosus* was a fast growing bacteria which multiplies once every 20 minutes, then little to *no* desired fermentation product could be expected. Thus, it is imperative that a biologically pure culture of *S. vellosus* be used in order to obtain the desired fermentation product using the controlled fermentation conditions disclosed in application Serial No. 477,766.

To maintain the biological purity of a culture during the propagation and fermentation stages, it is essential that operations and equipment be such that contaminants do not gain access into the system. Maintenance of sterile conditions to this extent is a major factor to the successful operation of a controlled fermentation operation.

It is clear to me that the "biologically pure culture" of Claim 5 is a product of a microbiologist and *not* a product of nature.

FURTHER, deponent sayeth not.

/s/ THOMAS L. MILLER
Dr. Thomas L. Miller

* * * *

PETITION OF APPEAL

TO THE U.S. COURT OF CUSTOMS AND PATENT APPEALS:

Applicants for patent, Malcolm E. Bergy, John H. Coats, and Vedpal S. Malik, residents of the County of Kalamazoo, State of Michigan, respectfully petition this Honorable Court for review of a decision by the United States Patent and Trademark Office Board of Appeals that the subject matter of claim 5 of their application is unpatentable under 35 U.S.C. 101 on the basis that the claim is directed to non-statutory subject matter.

Applicants respectfully reaffirm their original Oath, executed June 6, 1974, that they believe themselves to be the original and first inventors of the new process.

A complete application for patent was duly filed in the United States Patent and Trademark Office on June 10, 1974, in accordance with the Law, and the application was assigned Serial No. 477,766.

Subsequently, the Patent and Trademark Office Examiner rejected claim 5 under 35 U.S.C. 101 as drawn to non-statutory subject matter. The remaining claims 1-4 were indicated as allowable.

The Examiner's Final Rejection was appealed to the Board of Appeals which affirmed the rejection with one Board member dissenting.

By correspondence dated July 1, 1976, the applicants notified the Commissioner of Patents and Trademarks that the affirmance of the Examiner's rejection of claim 5 by the Board of Appeals was being appealed to this Honorable Court. The Notice to the Commissioner specified applicants' reasons of appeal.

By correspondence dated July 1, 1976, the applicants ordered a certified copy of the record from the Commissioner of Patents and Trademarks to be prepared and transmitted to this Honorable Court. The record will include the Notice and Reasons of Appeal.

The appeal fee of fifty ($50.00) dollars is enclosed.

Applicants respectfully assert that the foregoing facts entitle them to an appeal before this Honorable Court; and they respectfully pray that their appeal will be heard upon and for the reasons assigned therefor.

MALCOLM E. BERGY
JOHN H. COATS
VEDPAL S. MALIK

BOARD OF APPEALS

Serial No. 260,563

Docket No. 77-535

Filed December 28, 1976

IN THE MATTER OF THE APPLICATION OF
ANANDA M. CHAKRABARTY

Counsel for appellant:

Leo I. MaLossi; Joseph B. Forman, Frank L. Neuhauser

Commissioner of Patents and Trademarks

DOCKET ENTRIES

DATE	PROCEEDINGS AND ORDERS
March 14, 1977	Motion of appellant to extend time for filing printed transcript to April 27, 1977, filed.
March 15, 1977	Above motion granted.
April 26, 1977	PRINTED TRANSCRIPT OF RECORD, filed.
June 2, 1977	BRIEF FOR APPELLANT, filed.
July 11, 1977	Motion to extend time for filing brief by Commissioner of patents, not to extend beyond September 23, 1977, filed.
July 18, 1977	Response by appellant to Motion by Commissioner for extension of time for filing brief, filed.
July 21, 1977	Above motion granted. The extension of time not to extend beyond September 23, 1977.
September 23, 1977	BRIEF FOR COMMISSIONER OF PATENTS & TRADEMARKS, filed.
September 28, 1977	Motion by appellant to extend time to October 24, 1977, in which to file reply brief, filed.

DATE	PROCEEDINGS AND ORDERS
September 28, 1977	Above motion granted.
October 14, 1977	Motion of appellant to extend time for filing reply brief to October 31, 1977, filed.
October 14, 1977	Above motion granted.
October 20, 1977	REPLY BRIEF FOR APPELLANT, filed.
November 4, 1977	Motion of appellant for an additional 30 minutes for oral argument, filed.
November 7, 1977	Above motion denied. Court requests counsel to file an additional brief directed to *Ex parte Bergy* on or before November 28, 1977 (25 copies in typewritten form).
November 28, 1977	BRIEF FOR APPELLANT ON REQUEST BY COURT, filed.
November 28, 1977	BRIEF FOR COMMISSIONER OF PATENTS & TRADEMARK ON REQUEST BY COURT, filed.
December 5, 1977	Argued
March 2, 1978	Reversed, Rich, Judge. Concurring opinion by Chief Judge Markey. Dissenting opinion by Judge Baldwin. Dissenting opinion by Judge Miller.
March 24, 1978	FINAL MANDATE ISSUED TO THE COMMISSIONER OF PATENTS AND TRADEMARKS
July 26, 1978	Petition for Certiorari in Supreme Court of the U.S. No. 78-145.
Aug. 3, 1978	Petition to recall mandate, vacate decision and issue a new decision affirming the Board of Appeals, filed by Commissioner of Patents.
Aug. 11, 1978	Opposition to petition to recall mandate, vacate decision and issue new decision affirming the Board of appeals, filed.

DATE	PROCEEDINGS AND ORDERS

August 11, 1978 Above petition granted to extent that judgment is vacated, mandate recalled, and appeal restored to the calendar for hearing on November 6, 1978. Supplemental briefs to be filed as follows:

Appellant's Brief due on or before September 20, 1978. Commissioner's Brief due on or before October 20, 1978. Appellant's Reply Brief due on or before November 3, 1978.

September 18, 1978 SUPPLEMENTAL BRIEF FOR APPELLANT, filed.

September 18, 1978 BRIEF FOR AMICUS CURIAE FOR UNIVERSITY OF CALIFORNIA, filed.

September 18, 1978 Consent of Appellant and Commissioner of Patents to University of Calif. to file brief Amicus Curiae, filed.

September 20, 1978 BRIEF FOR AMICUS CURIAE FOR GENETECH, INC., filed.

September 20, 1978 BRIEF FOR AMICUS CURIAE FOR AMERICAN PATENT LAW ASS., filed.

October 19, 1978 Motion to Cornell D. Cornish for leave to file brief amicus curiae, filed.

October 19, 1978 Above motion granted, provisions of Rule 5.8 waived.

October 19, 1978 BRIEF FOR AMICUS CURIAE FOR CORNELL D. CORNISH AND THE VILLAGE OF BELLE TERRE, filed.

October 20, 1978 BRIEF FOR COMMISSIONER OF PATENTS & TRADEMARKS, filed.

October 27, 1978 Motion of Cornell D. Cornish and The Village of Belle Terre for oral argument as amicus curiae, filed.

October 27, 1978 Above motion denied.

DATE	PROCEEDINGS AND ORDERS

November 6, 1978 Argued

March 29, 1979 Reversed, Rich, J. Concurring opinion by Judge Baldwin. Dissenting opinion by Judge Miller.

April 20, 1979 FINAL MANDATE ISSUED TO THE COMMISSIONER OF PATENTS AND TRADEMARKS

July 27, 1979 Petition for writ of certiorari filed in the Supreme Court of the United States; No. 79-136.

October 29, 1979 Petition for Writ of Certiorari, granted.

APPLICATION OF ANADA M. CHAKRABARTY, FILED JUNE 7, 1972, SERIAL NUMBER 260,563, FOR MICROORGANISMS HAVING MULTIPLE, COMPATIBLE DEGRADATIVE ENERGY-GENERATING PLASMIDS AND PREPARATION THEREOF

Abstract of the Disclosure

Unique microorganisms have been developed by the application of genetic engineering techniques. These microorganisms contain at least two stable (compatible) energy-generating plasmids, these plasmids specifying separate degradative pathways. The techniques for preparing such multi-plasmid strains from bacteria of the genus *Pseudomonas* are described. Living cultures of two strains of *Pseudomonas* (*P. aeruginosa* [NRRL B-5472] and P. putida [NRRL B-5473]) have been desposited with the United States Department of Agriculture, Agricultural Research Service, Northern Marketing and Nutrient Research Division, Peoria, Illinois. The *P. aeruginosa* NRRL B-5472 was derived from *Pseudomonas aeruginosa* strain 1c by the genetic transfer thereto, and containment therein, of camphor, octane, salicylate and naphthalene degradative pathways in the form of plasmids. The *P. putida* NRRL B-5473 was derived from *Pseudomonas putida* strain PpG1 by genetic transfer thereto, and containment therein, of camphor, salicylate and naphthalene degradative pathways and drug resistance factor RP-1, all in the form of plasmids.

BACKGROUND OF THE INVENTION

The terminology of microbial genetics is sufficiently complicated that certain definitions will be particularly useful in the understanding of this invention:

Extrachromosomal element . . . a hereditary unit that is physically separate from the chromosome of the cell; the terms "extrachromosomal element" and "plasmid" are synonymous; when physically separated from the chromosome, some plasmids can be transmitted at high frequency to other cells, the transfer being without associated chromosomal transfer;

Episome . . . a class of plasmids that can exist in a state of integration into the chromosome of their host cell or as an autonomous, independently replicating, cytoplasmic inclusion;

Transmis—ible plasmid . . . a plasmid that carries genetic determinants for its own intercell transfer via conjugation;

DNA . . . deoxyribonucleic acid;

Bacteriophage . . . a particle composed of a piece of DNA encoded and contained within a protein head portion and having a tail and tail fibers composed of protein;

Transducing phage . . . a bacteriophage that carries fragments of bacterial chromosomal DNA and transfers this DNA on subsequent infection of another bacterium;

Conjugation . . . the process by which a bacterium establishes cellular contact with another bacterium and the transfer of genetic material occurs;

Curing . . . the process by which selective plasmids can be eliminated from the microorganism;

Curing agent . . . a chemical material or a physical treatment that enhances curing;

Genome . . . a combination of genes in some given sequence;

Degradative pathway . . . a sequence of enzymatic reactions (e.g. 5 to 10 enzymes are produced by the microbe) converting the primary substrate to some simple common metabolite, a normal food substance for microorganisms;

(Sole carbon source)—. . . indicative of a mutant incapable of growing on the given sole carbon source;

(Plasmid) del . . . indicative of cells from which the given plasmid has been completely driven out by

curing or in which no portion of the plasmid ever existed;

(Plasmid) . . . indicative of cells lacking in the given plasmid; or cells harboring a non-functional derivative of the given plasmid;

(Amino-acid)— . . . indicative of a strain that cannot manufacture the given amino acid;

(Vitamin)— . . . indicative of a strain that cannot manufacture the given vitamin and

(Plasmid)+ . . . indicates that the cells contain the given plasmid.

Plasmids are believed to consist of double-stranded DNA molecules. The genetic organization of a plasmid is believed to include at least one replication site and a maintenance site for attachment thereof to a structural component of the host cell. Generally, plasmids are not essential for cell viability.

Much work has been done supporting the existence, functions and genetic organization of plasmids. As is reported in the review by Richard P. Novick "Extrachromosomal Inheritance in Bacteria" (Bacteriological Reviews, June 1969, pp. 210-263, [1969]) on page 229, "DNA corresponding to a number of different plasmids has been isolated by various methods from plasmid-positive cells, characterized physicochemically and in some cases examined in the electron microscope".

There is no recognition in the Novick review of the existence of energy-generating plasmids specifying degradative pathways. As reported on page 237 of the Novick review, of the known (non energy-generating) plasmids "Combinations of four or five different plasmids in a cell seem to be stable."

Plasmids may be compatible (i.e. they can reside stably in the same host cell) or incompatible (i.e. they are unable to reside stably in a single cell). Among the known plasmids, for example, are sex factor plasmids and drug-resistance plasmids.

Also, as stated on page 240 of the Novick review, "Cells provide specific maintenance systems or sites for plasmids.

It is thought that attachment of such sites is required for replication and for segregation of replicas. Each plasmid is matched to a particular maintenance site . . .". Once a plasmid enters a given cell, if there is no maintenance site available, because of prior occupancy by another plasmid, these plasmids will be incompatible.

The biodegradation of aromatic hydrocarbons such as phenol, cresols and salicylate has been studied rather extensively with emphasis on the biochemistry of these processes, notably enzyme characterization, nature of intermediates involved and the regulatory aspects of the enzymic actions. The genetic basis of such biodegradation, on the other hand, has not been as thoroughly studied because of the lack of suitable transducing phages and other genetic tools.

The work of Chakrabarty and Gunsalus (Genetics, *68*, No. 1, page S10, [1971] has showed that the genes governing the synthesis of the enzymes responsible for the degradation of camphor constitute a plasmid. Similarly, this work has shown the plasmid nature of the octane-degradative pathway. However, attempts by the authors to provide a microorganism with both CAM and OCT plasmids were unsuccessful, these plasmids being incompatible.

In *Escherichia coli* artificial, transmissible plasmids (one per cell) have been made, each containing a degradative pathway. These plasmids, not naturally occurring, are F'*lac* and F'*gal*, wherein the lactose-and galactose-degrading genes were derived from the chromosome of the organism. Such plasmids are described in "F-prime Factor Formation in *E. Coli* K12" by J. Scaife (Genet. Res. Cambr. [1966], *8*, pp. 189-196).

If the development of microorganisms containing multiple compatible energy-generating plasmids specifying preselected degradative pathways could be made possible, the economic and environmental impact of such an invention would be vast. For example, there would be immediate application for such versatile microbes in the production of proteins from hydrocarbons ("Proteins from Petroleum"—Wang, Chemical Engineering, August 26, 1968, page 99); in cleaning up oil spills ("Oil Spills: An

Environmental Treat"—Environmental Science and Technology, Volume 4, February 1970, page 97); and in the disposal of used automotive lubricating oils ("Waste Lube Oils Pose Disposal Dilemma", Environmental Science and Technology, Volume 6, page 25, January 1972).

SUMMARY OF THE INVENTION

A transmissible plasmid has been found that specifies a degradative pathway for salicylate [SAL], an aromatic hydrocarbon. In addition, a plasmid has been identified that specifies a degradative pathway for naphthalene [NPL], a polynuclear aromatic hydrocarbon. The NPL plasmid is also transmissible.

Having established the existence of (and transmissibility of) plasmid-borne capabilities for specifying separate degradative pathways for salicylate and naphthalene, unique single-cell microbes have been developed containing various stable combinations of the [CAM], [OCT], [SAL], and [NPL] plasmids. In addition, stable combinations in a single cell of the aforementioned plasmids together with a non energy-generating plasmid [drug resistance factor RP-1] have been achieved. The versatility of these novel microorganisms has been demonstrated by the substantial extent to which degradation of such complex hydrocarbons as crude oil and Bunker C oil has been achieved thereby.

BRIEF DESCRIPTION OF THE DRAWING

The exact nature of the invention as well as objects and advantages thereof will be readily apparent from consideration of the following specification relating to the annexed drawing in which:

Fig. 1 shows the increase in growth rate in crude oil of *Pseudomonas* strain bacteria provided with increasing numbers of energy-generating degradative plasmids by the practice of this invention and

Fig. 2 shows the increase in growth rate in Bunker C oil of *Pseudomonas* strain bacteria provided with increasing numbers of energy-generating degradative plasmids by the practice of this invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Microorganisms prepared by the genetic engineering processes described herein are exemplified by cultures now on deposit with the United States Department of Agriculture. These cultures are identified as follows:

> *Pseudomonas aeruginosa* (NRRL B-5472) . . . derived from *Pseudomonas aeruginosa* strain 1c (ATCC No. 15692) by genetic transfer thereto, and containment therein, of camphor, octane, salicylate and naphthalene degradative pathways in the form of plasmids.
>
> *Pseudomonas putida* (NRRL B-5473) . . . derived from *Pseudomonas putida* strain PpGl (ATCC No. 17453) by genetic transfer thereto, and containment therein, of camphor, salicylate and naphthalene degradative pathways and a drug resistance factor RP-1, all in the form of plasmids. The drug resistance factor is responsible for resistance to neomycin/kanamycin, carbenicillin and tetracycline.

A sub-culture of each of these strains can be obtained from the permanent collection of the Northern Marketing and Nutrient Research Division, Agricultural Service, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

Morphological observations in various media, growth in various media, general group characterization tests, utilization of sugars and optimum growth conditions for the strains from which the above-identified organisms were derived are set forth in "The Aerobic Pseudomonads: A Taxonomic Study" by Stanier, R. Y. et al [Journal of General Microbiology *43*, pp. 159-271 (1966)]. The taxonomic properties of the above-identified organisms remain the same as those of the parent strains. *P. aeruginosa* strain 1c (ATCC No. 15692) is the same as strain 131 (ATCC No. 17503) in the Stanier et al study.

Later the designation for this strain was changed to *P. aeruginosa* PAO [Holloway, B. W. "Genetics of Pseudomonas", Bacteriological Reviews *33*, 419-443 (1969)]. *P. putida* strain PpG1 (ATCC No. 17453) is the same as strain 77 (ATCC No. 17453) in the Stanier et al study.

As will be described in more detail hereinbelow, these organisms thrive on a very wide range of hydrocarbons including crude oil and Bunker C oil. These organisms are non-pathogenic as is the general case with laboratory strains of *Pseudomonas*.

In brief, the process for preparing microbes containing multiple compatible energy-generating plasmids specifying separate degradative pathways is as follows:

1) selecting the complex or mixture to be degraded;

2) identifying the plurality of degradative pathways required in a single cell to degrade the several components of the complex or mixture therewith;

3) isolating a strain of some given microorganism on one particular selective substrate identical or similar to one of the several components (the selection of the microorganism is generally on the basis of a demonstrated superior growth capability);

4) determining whether the capability of the given strain to degrade the selective substrate is plasmid-borne;

5) attempting to transfer this first degradative pathway by conjugation to other strains of the same organism (or to the same strain which has been cured of the pathway) and then verifying the transmissible nature of the plasmid;

6) purifying the conjugatants (recipients of the plasmids by conjugation) and checking for distinctive characteristics of the recipient to insure that the recipient did, in fact, receive the degradative pathway;

7) repeating the process so as to introduce a second plasmid to the conjugatants;

8) rendering the first and second plasmids compatible, if necessary, by fusion of the plasmids and

9) repeating the process as outlined above until the full complement of degradative pathways desired in a single cell has been accomplished by plasmid transfer (and fusion, when required).

In the first reported instance (Chakrabarty et al article mentioned hereinabove) in which the attempt was made to locate more than one energy-generating degradative pathway in the same cell, it was found that CAM and OCT plasmids cannot exist stably under these conditions. In spite of the implication from these results that multiple energy-generating plasmid content in a single cell could be achieved but not maintained, it was decided to attempt to discover some way in which to overcome this problem of plasmid incompatability. As noted hereinabove and described more fully hereinbelow with specific reference to energy-generating plasmid transfer in the genus *Pseudomonas*, the problem of plasmid instability has now been solved by bringing about fusion of the plasmids in the recipient cell.

The development of single cell capability for the degradation and conversion of complex hydrocarbons was selected as the immediate beneficial application with particular emphasis on the genetic control of oil spills by the use of a single strain of *Pseudomonas*. In order to be able to cope with crude oil and Bunker C oil spills it was decided that the single cell of *Pseudomonas* derivate produced by this invention should possess degradative pathways for linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic hydrocarbons. *Pseudomonas aeruginosa* (NRRL B-5472) strain, which displays these degradative capabilities was thereupon eventually developed.

Massive oil spills that are not promptly contained and cleaned up have a catastrophic effect on aquatic lives. Microbial strains are known that can decompose individual components of crude oil (thus, various yeasts can degrade aliphatic straight-chain hydrocarbons, but not most of the aromatic and polynuclear hydrocarbons). *Pseudomonas* and other bacteria species are known to de-

grade the aliphatic, aromatic and polynuclear aromatic hydrocarbon compounds, but, unfortunately any given strain can degrade only a particular component. For this reason, prior to the instant invention, biological control of oil spills had involved the use of a mixture of bacterial strains, each capable of degrading a single component of the oil complex on the theory that the cumulative degradative actions would consume the oil and convert it to cell mass. This cell mass in turn serves as food for aquatic life. However, since bacterial strains differ from one another in a) their rates of growth on the various hydrocarbon components, b) nutritional requirements, production of antibiotics or other toxic material, and c) requisite pH, temperature and mineral salts, the use of a mixed culture leads to the ultimate survival of but a portion of the initial collection of bacterial strains. As a result, when a mixed culture of hydrocarbon-degrading bacteria are deposited on an oil spill the bulk of the oil often remains unattacked for a long period of time (weeks) and is free to spread or sink.

By establishing that SAL and NPL degradative pathways are specified by genes borne by transmissible plasmids in *Pseudomonas* and by the discovery that plasmids can be rendered stable (e.g. CAM and OCT) by fusion of the plasmids it has been made possible, for the first time, to genetically engineer a strain of *Pseudomonas* having the single cell capability for multiple separate degradative pathways. Such a strain of microbes equipped to simultaneously degrade several components of crude oil can degrade an oil spill much more quickly (days) than a mixed culture meanwhile bringing about coalescence of the remaining portions into large drops. This action quickly removes the opportunity for spreading of the oil thereby enhancing recovery of the coalesced residue.

Preparation of *P. aeruginosa* [NRRL B-5472]

The compositions of the synthetic mineral media for growth of the cultures were the same for all the *Pseudomonas* species employed. The mineral medium was prepared from:

PA Concentrate ..... 100 ml of 1 Molar K_2HPO_4
50 ml of 1 Molar KH_2PO_4
160 ml of 1 Molar NH_4Cl

100 X Salts ..... 19.5 gm $MgSO_4$
5.0 gm $MnCO_4 \cdot H_2O$
5.0 gm $FeSO_4 \cdot 7H_2O$
0.3 gm $CaCl_2 \cdot 2H_2O$
1.0 gm Ascorbic acid
1 liter H_2O

Each of the above (PA Concentrate and 100 X Salts) was sterilized by autoclaving. Thereafter, one liter of the mineral medium was prepared as follows:

PA Concentrate .... 77.5 ml
100 X Salts .... 10.0 ml
Agar .... 15.0 gm
H_2O .... to one liter (The pH is adjusted to 6.8-7.0).

All experiments were carried out at 32°C unless otherwise stated.

It was decided that a very useful hydrocarbon degradation capability would be attained in a single *Pseudomonas aeruginosa* cell, if the degradative pathways for linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic hydrocarbons could be transferred thereto. *Pseudomonas aeruginosa* PAO was selected because of its high growth rate even at temperatures as high as 45°C. Four strains of *Pseudomonas* were selected having the individual capabilities for degrading n-octane (a linear aliphatic hydrocarbon), camphor (a cyclic aliphatic hydrocarbon), salicylate (an aromatic hydrocarbon) and naphthalene (a polynuclear aromatic hydrocarbon).

The specific strains of *Pseudomonas* able to degrade these hydrocarbons were then treated with curing agent to verify the plasmid-nature of each of these degradative pathways. Of the known curing agents (e.g. sodium dodecyl sulfate, urea, acriflavin, rifampicin, ethidium bromide, high temperature, mitomycin C, acridine orange etc.) most were unable to cure any of the degradative

pathways. However, it is found (Table I) that the degradative pathways of the several species could be cured with mitomycin C. Each of the *Pseudomonas* strains bearing the specified degradative pathways are known in the art:

a) CAM+ *P. putida* PpGl . . . Proc. Nat. Acad. Sci. (U.S.A.), *60*, 168 (1968)

b) OCT+ *P. oleovorans* . . . J. Biol. Chem. *242*, 4334 (1967)

c) SAL+ *P. putida* R-1 . . . Bacteriological Proceedings 1972 p. 60

d) NPL+ *P. aeruginosa* . . . Biochem. J. *91*, 251 (1964)

TABLE I

Strain	Degradative Pathway	Mitomycin C Concentration (μg/ml)	Frequency of Curing (Percent)
CAM+ P. putida PpG1	cyclic aliphatic hydrocarbon (camphor)	0	< 0.01
		10	5
		20	95
OCT+ P. oleovorans	aliphatic hydrocarbon (n-octane)	0	< 0.1
		10	1.0
		20	3.0
SAL+ P. putida R-1	aromatic hydrocarbon (salicylate)	0	< 0.1
		5	0.7
		10	3.0
		15	4.0
NPL+ P. aeruginosa	polynuclear aromatic hydrocarbon (naphthalene)	0	< 0.1
		5	0.5
		10	1.8

Curing degradative pathways from each strain with mitomycin C was accomplished by preparing several test tubes of L broth [Lennox E.S. (1955), Virology, *1*, 190] containing varying concentrations of mitomycin C and inoculating these tubes with suitable dilutions of early stationary phase cells of the given strain to give concentrations 10^4 to 10^5 cells/ml. These tubes were incubated on a shaker at 32° C. for 2-3 days. Aliquots from tubes that showed some growth were then diluted and plated on glucose minimal plates. After growth at 32°C for 24 hours, individual colonies were split and respotted on glucose-minimal and degradative pathway—minimal plates to give the proportion of CAM—, OCT—, SAL— and NPL— in order to determine the frequency of curing. It was, therefore, shown that in each instance the degradative pathway genes are plasmidborne.

Transductional studies with a number of point mutants in the camphor and salicylate pathways has suggested that the cured segments lost either the entire or the major portion the plasmid genes. The plasmid nature of the degradative pathways was also confirmed from evidence of their transmissibility by conjugation from one strain to another (Table II). Although the frequency of plasmid transfer varies widely with individual plasmids and although OCT plasmid cannot be transferred from *P. oleovorans* to *P. aeruginosa* PAO at any detectable frequency, most of the plasmids can nevertheless be transferred from one strain to another by conjugation.

The plasmid transfers, instead of being made to other strains could have been made to organisms of the same strain, that had been cured of the given pathway with mitomycin C, acridine orange or other curing agent.

Pseudomonas putida U has been described in the article by Feist et al [J. Bacteriology *100*, p. 869-877 (1969)].

The auxotropic mutants (mutants that require a food source containing a particular amino acid or vitamin for growth) shown in Table II as donors were each grown in a complex nutrient medium (e.g. L broth) to a population density of at least about 10^8 cells/ml without shaking in a period of from 6 to 24 hours. The prototropic (cells capable of growing on some given minimal source of carbon) recipients to which degradative pathway transfer was desired were grown separately in the same complex nutrient medium to a population density of at least about 10^8 cells/ml with shaking in a period of from 4 to 26 hours. For each degradative pathway transfer these cultures were mixed in equal volumes, kept for 15 minutes to 2 hours at 32°C without shaking (to permit conjugation to occur) and then plated on minimal plates containing the particular substrate as the sole source of carbon. This procedure for cell growth of donor and recipient and the mixing thereof is typical of the manner in which conjugation and plasmid transfer is encouraged in the laboratory, this procedure being designed to provide a very efficient transfer system. Temperature is not critical, but the preferred temperature range is 30-37°C. Reduction in the population density of either donor or recipient below about 1,000,000 cells/ml or any change in the optimal growth conditions (stationary growth of donor, agitated growth of recipient, growth in high nutrient content medium, harvest of recipient cells at log phase) will drastically reduce the frequency of plasmid transfer.

The details for preparing and isolating auxotropic mutants is described in the textbook, "The Genetics of Bacteria and Their Viruses" by William Hays [John Wiley & Sons, Inc. (1965)].

TABLE II

Donor	Recipient	Degradative Pathway	Frequency of Transfer
Trp$^-$ CAM$^+$ *P. putida* PpG1	*P. aeruginosa* PAO	CAM	10^{-3}
	CAM^{del} *P. putida*	CAM	10^{-2}
Met$^-$ OCT$^+$ *P. oleovorans*	*P. aeruginosa* PAO	OCT	$< 10^{-9}$
	P. putida PpG1	OCT	10^{-9}
	P. putida U	OCT	10^{-7}
His$^-$ SAL$^+$ *P. putida* R-1	*P. aeruginosa* PAO	SAL	10^{-7}
	P. putida PpG1	SAL	10^{-6}
Trp$^-$ NPL$^+$ *P. aeruginosa*	*P. putida* PpG1	NPL	10^{-7}
	NPL^{del} *P. aeruginosa* PAO	NPL	10^{-5}

Abbreviations:

Trp - tryptophane
Met - methionine
His - histidine

Control cultures of donors and recipients were also placed individually on minimal plates containing the requisite substrate in each instance as the sole source of carbon, to determine the reversion frequency of donor and recipient cells.

All plates (including controls) were incubated at 30-37°C for several days. In each instance in which colonies appeared in numbers exceeding the colony growth on the reversion plates, it was established that degradative pathway transfer had occurred between the donors and recipients. Such conjugatants were then purified by a series of single colony isolation cultures and checked for growth rates or other distinctive characteristics of the recipient to insure that the recipient actually received the given degradative pathway.

Having determined that the degradative pathways were plasmid-borne and transmissible, the task of transferring the multiplicity of plasmids to a single cell *P. aeruginosa* PAO was undertaken. Prior work (referred to hereinabove) had established that OCT plasmids could not be transferred from *P. oleovorans* to *P. aeruginosa* PAO. Therefore, the first task was to discover how (if at all) the OCT and CAM plasmids could be rendered compatible.

The CAM plasmid was transferred to a Met—mutant of OCT+*P. oleovorans* strain from a CAM+*P. putida* strain. The conjugatant is, of course, unstable and will segregate either CAM or OCT at an appreciable rate. Therefore, the conjugatant was alternatively grown in camphor and then octane as sole sources of carbon to isolate those cells in which both of these degradative pathways were present, even though unstable. The surviving cells were centrifuged, suspended in 0.9% saline solution and irradiated with UV rays (3 General Electric FS-5 lamps providing a total of about 24 watts). Aliquots were drawn from the suspension as follows: one aliquot was removed before UV treatment, one aliquot after UV exposure for 30 seconds and one aliquot after UV exposure for 60 seconds. These aliquots of irradiated cells were grown in the absence of light for 3 hours in L broth and were then used as donors for the transfer of plasmids to the *P. aeruginosa* PAO strain

as recipient, selection being made for the OCT plasmid on an octane minimal plate.

As is shown in Table III aliquots of similarly irradiated suspensions for Met—OCT+CAM ^{del} P. *oleovorans* and Met—CAM+OCT ^{del} *P. oleovorans* were prepared and used as plasmid donors to *P. aeruginosa* PAO, selection being made for the plasmids shown. The Met—CAM+ OCT ^{del} strain was prepared by introducing CAM plasmids into Met—OCT+ mutant of *P. oleovorans* and selecting for CAM+ conjugatants, which have lost the OCT plasmid. The Met—OCT+CAM ^{del} *P. oleovorans* is the Met— mutant of wild type *P. oleovorans.*

The failure to secure determinable transfer of OCT plasmids from Met—OCT+*P. oleovorans* to the recipient and the success in securing transfer of CAM plasmids from Met—CAM+OCT ^{del} *P. oleovorans* to the recipient are shown. These results support the theory that the successful transfer of OCT plasmids from the MET— CAM+ OCT+ *P. oleovorans* (that had been irradiated for 30 seconds with UV rays) to *P. aeruginosa* PAO had been made possible by the fusion of the CAM and OCT plasmids in the *P. oleovorans* by the UV exposure and the subsequent transfer of CAM/OCT plasmids in combination (with separate degradative pathways) to the recipient.

TABLE III.

Donor	Recipient	Selected Plasmid	Period of UV-Irradiation (Sec)	Transfer of Frequency
Met$^-$OCT$^+$ *P. oleovorans*	*P. aeruginosa* PAO	OCT	0	$< 10^{-9}$
			30	$< 10^{-9}$
			60	$< 10^{-9}$
		CAM	0	10^{-4}
			30	10^{-5}
			60	10^{-7}
Met$^-$CAM$^+$OCT^{del} *P. oleovorans*	*P. aeruginosa* PAO	OCT	0	$< 10^{-9}$
			30	10^{-8}
			60	$< 10^{-9}$
Met$^-$CAM$^+$OCT$^+$ *P. oleovorans*	*P. aeruginosa* PAO	CAM	0	10^{-4}
			30	10^{-5}
			60	10^{-7}

Table IV presents verification of this theory of co-transfer of CAM and OCT fused plasmids. A Trp— mutant of CAM+OCT+ *P. aeruginosa* PAO that had been provided with its multiple plasmids by the methods described herein for plasmid transfer and plasmid fusion was used as the donor. After conjugation between the donor and OCT ^{del} CAM ^{del} *P. putida* PpG1, the resulting culture was plated on minimal plates containing camphor and also on minimal plates containing n-octane. Part of each of 132 colonies growing on the CAM minimal plates were transferred to OCT minimal plates and part of each of 219 colonies growing on the OCT minimal plates were transferred to CAM minimal plates. Each of these transferred portions grew, which tends to establish that a) both CAM and OCT plasmids had been transferred to the conjugatant, b) the transfer had been on a one-for-one basis and, therefore, c) the CAM and OCT plasmids were fused together.

Similar plasmid transfer was carried out between the Trp—CAM+OCT+ *P. aeruginosa* PAO donor and OCT ^{del} CAM ^{del} *P. aeruginosa* PAO and similar selection procedures were employed. The results further reinforced the above position as to the fused nature of the transferred CAM and OCT plasmids. When the CAM and OCT plasmids have been subjected to UV radiation as disclosed, if either CAM or OCT plasmid is transferred, the other plasmid will always be associated with it regardless of which plasmid is selected first. If either plasmid of the fused pair is cured from the cell, both plasmids are lost simultaneously. Thus, the conjugatants were treated with mitomycin C and the resultant CAM ^{del} segregants were examined. Invariably all CAM ^{del} segregants were found to have lost the OCT plasmid as well. Thus, the facts of simultaneous curing of the two plasmids and the co-transfer thereof strongly suggests that incompatible plasmids treated with means for cleaving the DNA of the plasmids results in fusion of the DNA segments to become part of the same replicon.

TABLE IV

Donor	Recipient	Selected Plasmid	Non-selected Plasmid	Total $^{+}$ OCT^{+}/CAM^{+}
Trp^{-}CAM^{+}OCT^{+}	OCT^{del}CAM^{del}	CAM	OCT	132/132
		OCT	CAM	219/219
P. aeruginosa PAO	*P. putida* PpG1	CAM	OCT	107/107
	OCT^{del} CAM^{del}	OCT	CAM	96/96
	P. aeruginosa PAO			

Having successfully overcome all obstacles to the formation of a stable CAM+OCT+SAL+NPL+ *Pseudomonas* the several energy-generating degradative plasmids were transferred to a single cell as is shown in Table V by the conjugation techniques described hereinabove. The initial *P. aeruginosa* strain used is referred to herein as *P. aeruginosa* PAO, formerly known as *P. aeruginosa* strain 1c available as ATCC No. 15692 and/or ATCC No. 17503. This strain of *P. aeruginosa* does not contain any known energy-generating plasmid. The CAM and OCT plasmids exist in the fused state, are individually and simultaneously functional and appear perfectly compatible with the individual compatible SAL and NPL plasmids. Tests for compatibility of both CAM+ OCT+SAL+ *P. aeruginosa* PAO and CAM+ OCT+ SAL+ NPL+ *P. aeruginosa* PAO revealed that there is no segregation of the plasmids in excess of that found in the donor. Plasmids will be accepted and maintained by *P. acidovorans*, *P. alcaligenes* and *P. fluorescens*. All of these plasmids should be transferable to and maintainable in these and many other species of *Pseudomonas*, such as *P. putida*, *P. oleovorans*, *P. multivorans*, etc.

Superstrains such as the CAM+OCT+SAL+NPL+ strain of *P. aeruginosa* PAO can grow on a minimal plate of any of camphor, n-octane, salicylate, naphthalene and, because of the phenomenon of relaxed specificity, on compounds similar thereto. Thus, the effectiveness of a given degradative plasmid does not appear to be diminished in its ability to function singly by the presence of other degradative plasmids in the same cell.

TABLE V

Donor	Recipient	Selected Plasmid	Phenotype of the Conjugatant
$Trp^-CAM^+OCT^+$ *P. aeruginosa* PAO	*P. aeruginosa* PAO	CAM	CAM^+OCT^+ *P. aeruginosa* PAO
His^-SAL^+ *P. putida* R-1	CAM^+OCT^+ *P. aeruginosa* PAO	SAL	$CAM^+OCT^+SAL^+$ *P. aeruginosa* PAO
Trp^-NPL^+ *P. aeruginosa*	$CAM^+OCT^+SAL^+$ *P. aeruginosa* PAO	NPL	$CAM^+OCT^+SAL^+NPL^+$ *P. aeruginosa* PAO

Indication of the capability of all degradative plasmids to function simultaneously in energy generation is provided by tests in which CAM+OCT+SAL+NPL+ *P. aeruginosa* PAO superstrain was added to separate broth samples each of which contained 1 millimolar (mM) of nutrient (a suboptimal concentration), one set of samples containing camphor, a second set of samples containing n-octane, a third set of samples containing salicylate and a fourth set of samples containing naphthalene, these being the sole sources of carbon in each instance. The superstrain grew very slowly in the separate sole carbon source samples. However, when the superstrain was added to samples containing all four sources of carbon present together in the same (1 mM) concentration of 4 mM, the rate of growth increased considerably establishing that simultaneous utilization of all four sources of carbon had occurred.

Next, the ability of such superstrains to degrade crude oil was demonstrated. Crude oils, of course, vary greatly (depending upon source, period of activity of the well, etc.) in the relative amount of linear aliphatic, cyclic aliphatic, aromatic and polynuclear hydrocarbons present, although some of each of these classes of hydrocarbons is typically present in some amount in the chemical make up of all crude oils from producing wells.

Fig. 1 shows the difference in growth capabilities in crude oil as the sole source of carbon of four single cell strains of *P. aeruginosa* PAO. Curve *a* shows the cell growth as a function of time of *P. aeruginosa* without any plasmid-borne energy-generating degradative pathways. Curve *b* shows greater cell growth as a function of time for SAL+ *P. aeruginosa*. Curve *c* shows still greater cell growth as a function of time for SAL+ NPL+ *P. aeruginosa*. Curve *d* shows cell growth that is significantly greater still as a function of time for the CAM+OCT+SAL+NPL+superstrain of *P. aeruginosa*. These results clearly establish that cells artifically provided by the practice of this invention with the genetic capability for degrading different hydrocarbons can grow at a faster rate and better on crude oil as the plasmid-borne degradative pathways are increased in number and

variety, because of the facility of these degradative pathways to simultaneously function at full capacity.

Similar results are shown in Fig. 2 displaying the growth capabilities of this same series of organisms utilizing Bunker C oil as the sole source of carbon. Bunker C is (or is prepared from) the residuum remaining after the more commercially useful components have been removed from crude oil. This residuum is very thick and sticky and without significant use, per se. A small amount of volatile hydrocarbons is often added thereto to lower the viscosity. Curve *r* reflects the cell growth as a function of time of *P. aeruginosa* cells not having any plasmid-borne energy-degradative pathways. Curve *s* shows increased cell growth as a function of time for SAL+ *P. aeruginosa*. Curve *t* shows further increase in cell growth as a function of time for SAL+ NPL+ P. *aeruginosa*. Curve *u* shows still more significant cell growth as a function of time for CAM+ OCT+ SAL+ NPL+ *P. aeruginosa*.

The SAL+ *P. aeruginosa* and SAL+ NPL+ *P. aeruginosa* cultures were prepared as shown in Table VI below:

TABLE VI

Donor	Recipient	Selected Plasmid	Conjugatant
His^-SAL^+ *P. putida* R-1	*P. aeruginosa* PAO	SAL	SAL^+ *P. aeruginosa* PAO
Trp^-NPL^+ *P. aeruginosa*	SAL^+ *P. aeruginosa* PAO	NPL	SAL^+NPL^+ *P. aeruginosa* PAO

The experiments providing the data for Figs. 1 and 2 were conducted in 250 ml Erlenmeyer flasks. To each flask was added 50 ml of mineral medium (described hereinabove) with pH adjusted to 6.8—7.0; 2.5 ml of the sole carbon source (crude oil or Bunker C) and 5 x 10^6—1 x 10^7 cells. Growth was conducted at 32°C with shaking. At daily intervals 5 ml aliquots were taken. The optical densities of these aliquots were determined at 660 nm in a Bausch & Lomb, Inc. colorimeter to determine organism density. Also, viable cell counts were determined by diluting portions of the aliquots and plating on L-agar (L-broth containing agar) plates. The colonies were counted after 24 hours of incubation at 32°C and these counts were used to construct Figs. 1 and 2. Also, the cells were submitted to protein analysis, to be discussed hereinbelow.

The 2.5 ml of crude oil or Bunker C appears to have initially offered an essentially unlimited food supply, but the results shown may well represent less than the full capability of the superstrain, because the relative amounts of the various hydrocarbons (degradable by the CAM+, OCT+, SAL+ and NPL+ plasmids) present in the carbon sources had not been ascertained and after a couple of days the food supply for one or more plasmids may have been limited.

A very significant aspect of the growth of the superstrain in crude and Bunker C oils is the fact that the components, which would spread the quickest on the water's surface from spills of these oils, disappear within 2-3 days and the remaining components of the oil coalesce to form large droplets that cannot spread out. These droplets can be removed more easily by mechanical recovery techniques as the microbes continue to consume these remaining components.

In practice in inoculum of dry (or lyophilized) powders of these genetically engineered microbes will be dispersed over (e.g. from overhead) an oil spill as soon as possible to control spreading of the oil, which is so destructive of marine flora and fauna and the microbes will degrade as much of the oil as possible to reduce the amount that need be recovered mechanically, when equipment has

reached the scene and has been rendered operative. A particularly beneficial manner of depositing the inoculum on the oil spill is to impregnate straw with the inoculum and drop the inoculated straw on the oil spill where both components will be put to use—the inoculum (mass of microbes) to degrade the oil and the straw to act as a carrier for the microbes and also to function as an oil absorbent. Other absorbent materials may be used, if desired, but straw is the most practical. No special care need be taken in the preparation and storage of the dried inoculum or straw (or other absorbent material) coated with inoculum. No additional nutrient or mineral content need be supplied. Also, although culture from the logarithmic growth phase is preferred, culture from either the early stationary or logarithmic growth phases can be used.

It is reasonable to expect that a vast number of plasmid-borne hydrocarbon degradative pathways remain undiscovered. Hopefully, now that a method for controlled genetic additions to the natural degradative capabilities of microbes has been demonstrated by this invention, still more new and useful single cell organisms can be prepared able to degrade even more of the large number of hydrocarbons in crude oil, whether or not the plasmids yet to be found are compatible with each other or with those plasmids present in superstrains NRRL B-5472 and NRRL B-5473.

Both of these superstrains can be used as recipients for more plasmids. The capability for utilizing fusion (by UV irradiation or X-ray exposure) to render additional plasmids compatible is actually increased in a multiplasmid conjugatant, because of the larger selection of stable plasmids to which the newly introduced plasmid can be fused.

Preparation of *P. putida* [NRRL B-5473]

The mineral medium and the technique for fostering conjugation was the same as described above. A culture of antibiotic-sensitive *P. putida* PpGl was cured of its CAM plasmids with mitomycin C and was used as the

initial recipient. This strain of *P. putida* is sensitive to small (e.g. 25 micrograms/ml) concentrations of neomycin/kanamycin, carbenicillin and tetracycline. As is shown in Table VII below, all the doner strains are auxotropic mutants, because the use of auxotropic mutant doners facilitates counterselection of the conjugatants due to the ease of selecting against such donors.

TABLE VII

Donor	Recipient	Selected Plasmid	Phenotype of the Conjugatant
Trp$^-$CAM$^+$ *P. putida* PpG1	CAM^{del} *P. putida* PpG1	CAM	CAM$^+$ *P. putida* PpG1
His$^-$SAL$^+$ *P. putida* R-1	CAM$^+$ *P. putida* PpG1	SAL	CAM$^+$SAL$^+$ *P. putida* PpG1
Trp$^-$NPL$^+$ *P. aeruginosa*	CAM$^+$SAL$^+$ *P. putida* PpG1	NPL	CAM$^+$SAL$^+$NPL$^+$ *P. putida* PpG1
Met$^-$ *P. aeruginosa* Strain 1822 (RP-1)	CAM$^+$SAL$^+$NPL$^+$ *P. putida* PpG1	RP-1	CAM$^+$SAL$^+$NPL$^+$RP-1$^+$ *P. putida* PpG1

The *P. aeruginosa* RP-1 strain is disclosed in the Sykes et al article [Nature *226*, 952 (1970)]. Selection for the RP-1 plasmid was accomplished on a neomycin/kanamycin plate. Further, CAM+ SAL+ NPL+ RP-1+ *P. putida* PpG1 has been determined to be resistant to carbenicillin and tetracycline establishing that the RP-1 plasmid is actually present and that the organisms that survived the selection process were not merely the results of mutant development. Also, the plasmids of this superstrain can be transferred and can be cured. The rate of segregation (spontaneous loss) of plasmids from the superstrain has been found to be the same as in the donors.

Both superstrains can, of course, be used as a source of plasmids in addition to those sources disclosed herein. For example, to transfer CAM, SAL or NPL plasmids from CAM+ SAL+ NPL+ RP-1+ *P. putida* PpG1 to a given *Pseudomonas* recipient, the procedures for cell growth of donor and recipient and the mixing thereof for optimized conjugation is the same as described herein-above. These plasmids will have different frequencies of transfer at different times. The order of diminishing frequency of transfer is CAM, NPL, SAL. For the transfer of CAM plasmid, after conjugation, selection is made for CAM. Surviving colonies are subdivided and selection is made for SAL, NPL and CAM plasmids from each colony. Those protions surviving only on camphor as the sole source of carbon will have received the CAM plasmid free of the SAL or NPL plasmids. The same procedure can be followed for the individual transfer of SAL or NPL plasmids.

In addition to the previously discussed capability for improved treatment of oil spills, considerable improvement is now possible in the microbial single-cell synthesis of proteins from carbon-containing substrates. The restrictions of having to employ substantially single-component substrates, e.g., alkanes, paraffins, carbohydrates, etc. has now been removed, simultaneously providing the opportunity for increases of 50-100 fold in the amount of cell mass that may be produced by a single cell in a given time period, when the given single cell has been provided with multiple energy-generating plasmids. Also, being

able to optimize the protein production of bacteria is of particular interest since bacterial cell mass has a much greater protein content and most bacteria have greater tolerance for heat than yeasts. This latter aspect is of importance since less refrigeration is necessary to remove the heat generated by the oxidative degradation of the substrate.

The general process and apparatus for single cell production of protein is set forth in the Wang article (incorporated by reference) referred to hereinabove. One particular advantage of the multi-plasmid single cell organism of this invention is that after the cell mass has been harvested it can be subjected to a subsequent incubation period in a mineral medium free of any carbon source for a sufficient period of time to insure the metabolism of residual intra-cellular hydrocarbons, e.g. polynuclear aromatics which are frequently carcinogenic. Presently, treatment of cell mass to remove unattacked hydrocarbons often leads to reduction in the quality of the protein product.

The economics of protein production by single-cell organisms will be further improved by the practice of this invention, because of the reduced cost of substrate (e.g. oil refinery residue, waste lubricating oil, crude oil) utilizable by organisms provided with preselected plasmid content.

Cell mass grown in crude oil using NRRL B-5472 was harvested by centrifugation, washed two times in water and dried by blowing air (55°C) over the mass overnight. The dried mass was hydrolyzed and analyzed for amino acid content by the technique described "High Recovery of Tryptophane from Acid Hydrolysis of Proteins" Matsubara et al [Biochem. and Biophys. Res. Comm. 35 No. 2, 175-181 (1969)]. The amino acid analysis showed that the amino acid distribution of superstrain cell mass grown in crude oil is comparable to beef in threonine, valine, cystine, methionine, isoleucine, leucine, phenylalanine and tryptophane content and significantly superior to yeast in methionine content.

Continued capacity for increasing the degrading capability of the superstrains now on deposit has been made

possible by the practice of this invention as more plasmid-borne degradative pathways are discovered. To date *P. aeruginosa* strain 1822 has been provided with all four known hydrocarbon degradative pathways (OCT, CAM, SAL, NPL) plus the drug-resistance factor RP-1 found therein. If there is an upper limit to the number of energy-generating plasmids that will be received and maintained in a single cell, this limit is yet to be reached. Attempts to integrate plasmids (CAM, OCT, SAL) with the cell chromosome have been unsuccessful as indicated by failure to mobilize the chromosome. Such results have so far verified the extrachromosomal nature of the energy-generating and drug-resistance plasmids. There is, of course, no reason to expect that the only plasmids are those that specify degradative pathways for hydrocarbons. Conceivably plasmids may be discovered that will provide requisite enzyme series for the degradation of environmental pollutants such as insecticides, pesticides, plastics and other inert compounds.

Energy-generating plasmids in general are known to have broad inducer and substrate specificity [i.e. enzymes will be formed and will act on a variety of structurally similar compounds]. For example, the CAM plasmid is known to have a very relaxed inducer and substrate specificity [Gunsalus et al—Israel J. Med. Sci., *1*, 1099-1119 (1965) and Hartline et al—Journal of Bacteriology, *106*, 468-478 (1971)]. Similarly, the OCT plasmid has broad inducer and substrate specificity [Peterson et al—J. Biol. Chem. *242*, 4334 (1967)]. In the practice of the instant invention it has been demonstrated that plasmids display the same degree of relaxed specificity in the conjugatant as in the donor.

Thus, by the practice of this invention new facility and capability for growth has been embodied in useful single-cell organisms by the manipulation of phenomena that had been previously undiscovered (i.e. the plasmid-borne nature of the degradative pathways for salicylate and naphthalene) and/or had been previously unsuccessfully applied (i.e., rendering stable a plurality of previously incompatible plasmids in the same single cell).

Filed concurrently herewith is U.S. Application S.N. (RD-5561)—Chakrabarty, filed , 1972 and assigned to the assignee of the instant invention.

What I claim as new and desire to secure by Letters Patent of the United States is:

1. A single cell microorganism containing at least two stable energy-generating plasmids, said plasmids specifying separate degradative pathways.

2. The single cell microorganism of claim 1 wherein the pathways are hydrocarbon degradative pathways.

3. The single cell microorganism of claim 2 wherein the hydrocarbon degradative pathways are selected from the group consisting of linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic.

4. A bacterium containing at least two stable energy-generating plasmids, said plasmids specifying separate degradative pathways.

5. The bacterium of claim 4 wherein the pathways are hydrocarbon degradative pathways.

6 The bacterium of claim 5 wherein the hydrocarbon degradative pathways are selected from the group consisting of linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic.

* * * * *

for Claim 7, see Rejected Claim 7.

* * * * *

For Claims 8 and 9, see Rejected Claims 8 and 9.

10. The *Pseudomonas* bacterium of claim 7 wherein the pathways are hydrocarbon degradative pathways.

11. The *Pseudomonas* bacterium of claim 10, said bacterium being of the specie *P. aeruginosa.*

12. The *Pseudomonas* bacterium of claim 10, said bacterium being of the specie *P. putida.*

* * * * *

For Claim 13, see Rejected Claim 13.

14. The *Pseudomonas* bacterium of claim 13, said bacterium being of the specie *P. aeruginosa.*

* * * * *

For Claim 15, see Rejected Claim 15.

16. The *Pseudomonas* bacterium of claim 13, said bacterium being of the specie *P. putida.*

For Claim 17, see Rejected Claim 17.

18. An inoculum for the degradation of a preselected substrate, said inoculum consisting essentially of single cell microorganisms at least some of which contain at least two stable energy-generating plasmids, said plasmids specifying separate degradative pathways.

19. The inoculum of claim 18 wherein the degradative pathways in the microorganisms having multiple energy-generating plasmids are hydrocarbon degradative pathways.

20. The inoculum of claim 19 wherein the hydrocarbon degradative pathways are selected from the group consisting of linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic.

* * * * *

For Claim 21, see Rejected Claim 21.

22. The inoculum of claim 21 wherein the degradative pathways in the bacteria having multiple energy-generating plasmids are hydrocarbon degradative pathways.

23. The inoculum of claim 21 wherein the bacteria having multiple energy-generating plasmids are of the genus *Pseudomonas.*

* * * * *

For Claims 24 to 26 inclusive, see Rejected Claims 24 to 26 inclusive.

27. In the process in which a first energy-generating plasmid specifying a degradative pathway is transferred by conjugation from a donor single cell organism to a recipient single cell organism containing at least one energy-generating plasmid that is incompatible with said first plasmid, said transfer occurring in the quiescent state after the mixing of substantially equal volumes of cultures of said donor and said recipient, each culture presenting the respective organisms in a complex nutrient liquid medium at a population density of at least about 1,000,000 cells/ml, the improvement wherein after conjugation has occurred, the multi-plasmid conjugatant

organisms are subjected to DNA-cleaving radiation in a dosage sufficient to fuse the first plasmid and the plasmid incompatible therewith located in the same cell.

* * * * *

For Claim 28, see Allowed Claim 28.

29. The improvement of claim 28 wherein the single cell organisms are of the genus *Pseudomonas*, the first plasmid specifying the degradative pathway for camphor and the recipient *Pseudomonas* containing the degradative pathway for n-octane.

30. An inoculated medium for the degradation of floating liquid hydrocarbon substrate material, said inoculated medium comprising a buoyant carrier material and single cell microorganisms carried thereby, at least some of said microorganisms containing at least two stable energy-generating plasmids specifying separate hydrocarbon degradative pathways and said carrier material being able to absorb said hydrocarbon material.

* * * * *

For Claims 31 and 32, see Allowed Claims 31 and 32.

33. The inoculated medium of claim 32 wherein the multiplasmid containing single cell microorganisms are bacteria.

34. The inoculated medium of claim 33 wherein the bacteria are of the genus *Pseudomonas*.

35. The inoculated medium of claim 34 wherein the bacteria are of the specie *P. aeruginosa*.

36. The inoculated medium of claim 34 wherein the bacteria are of the specie *P. putida*.

DELCARATION, POWER OF ATTORNEY, AND PETITION

I, Ananda M. Chakrabarty declare that I am a citizen of India residing at Latham, New York; that I have read the foregoing specification and claims and I verily believe I am the original, first, and sole inventor of the invention in Microorganisms Having Multiple Compatible Degradative Energy-Generating Plasmids and Preparation Thereof described and claimed therein; that I do not know and do not believe that this invention was ever known or used before my invention thereof, or patented or described in any printed publication in any country before my invention thereof, or more than one year prior to this application; or in public use or on sale in the United States more than one year prior to this application; that this invention has not been patented in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application; and that no application for patent on this invention has been filed by me or my representatives or assigns in any country foreign to the United States, except as follows:

And I hereby appoint John F. Ahern, Charles T. Watts and Leo I. MaLossi, Corporate Research and Development, Bldg. K-1, General Electric Company, P.O. Box 8, Schenectady, New York 12301, and Frank L. Neuhauser, Reg. No. 14975, Oscar B. Waddell, Reg. No. 15415, and Joseph B. Forman, Reg. No. 15795, Washington Patent Operation, General Electric Company, 2001 Jefferson Davis Highway, Arlington, Virginia 22202, jointly, and each of them severally, my attorneys and attorney, with full power of substitution, delegation and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent and to transact all business in the Patent Office connected therewith. I hereby direct that all correspondence in connection with this application be addressed to the said John F. Ahern, Corporate Research and Development, Building K-1, General Electric Company, P.O. Box 8, Schenectady, New York 12301.

The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false

statements may jeopardize the validity of the application or any patent issuing thereon.

Wherefore I pray that Letters Patent be granted to me for the invention described and claimed in the foregoing specifications and claims, and I hereby subscribe my name to the foregoing specification and claims, declaration, power of attorney, and this petition, this 1 day of June, 1972.

Inventor ANANDA M. CHAKRABARTY
First Name Middle Initial Last Name
Post Office Address
31-11 Latham Village
Latham, New York 12110

PRINT OF DRAWINGS AS ORIGNALLY FILED

260563

FIG. 1

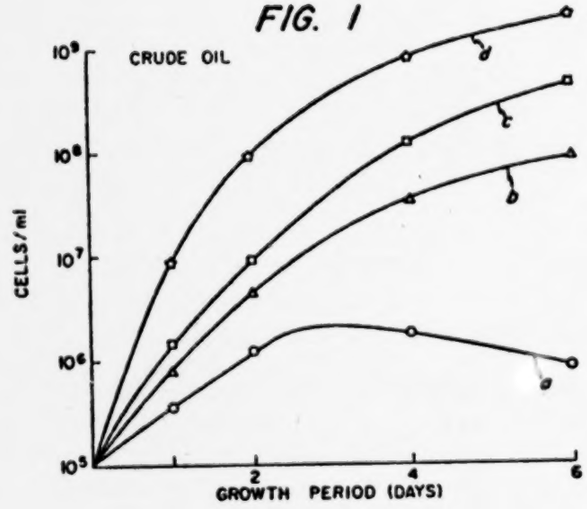
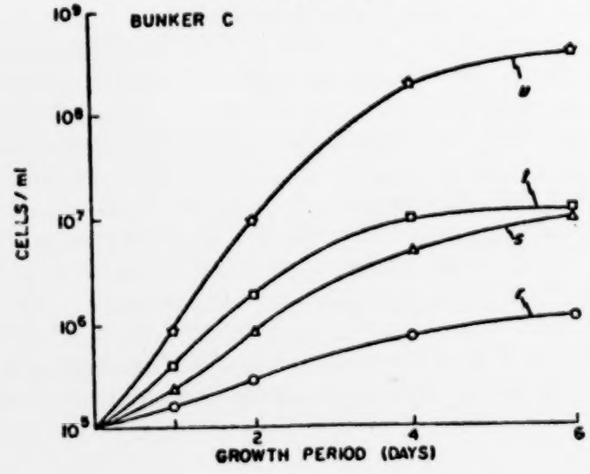


FIG. 2



AMENDMENT, DECEMBER 6, 1973

3

Honorable Commissioner of Patents,

SIR:

In response to the Office Action dated September 19, 1973, please amend the above-identified application as follows:

Page 1, line 16, delete "Transmisible" and
insert—Transmissible—

Page 25, line 13, delete "suggests" and insert —suggest—

Cancel claims 1-6, 10-12, 14, 16, 18-20, 22, 23 and 33.

For Claim 7, see Rejected Claim 7.

For Claim 21, see Rejected Claim 21.

Claim 34, line 1, delete "33" and insert —30—

REMARKS

The rejection of claims 7-9, 13, 15, 17, 21, 24-32 and 34-36 in the Office Action dated September 19, 1973 is respectfully traversed. Withdrawal of the grounds of rejection set forth therein is respectfully requested.

With respect to the rejection of claims 1-26 under 35 U.S.C. 101 as being nonstatutory, attention is respectfully directed to Section 706.03(a) Nonstatutory Subject Matter. In the example of "Naturally Occurring Article" the statement is made that "a thing occurring in nature, *which is substantially unaltered*, is not a 'manufacture'" (emphasis supplied). The organisms disclosed and claimed by Applicant are not different in *degree* from the natural characteristics of such organisms, but are different in *kind*, the natural organism having been made into one having a totally new capability.

Thus, in the case of spills of crude oil, it is important that the oil spill be degraded quickly with coalesce of the remaining portions into large drops whereby the opportunity for spreading of the oil over the water is obviated. As is explained in applicant's specification, page 11, line 6, through page 12, line 20, a mixed culture does not have the equivalent capability of a single organism having multiple degradative pathways.

As is pointed out in the Handbook of Patents by H. A. Toulmin, Jr. (The W. H. Anderson Company, 1948, page 40), "A patentable composition of matter may well result or be formed by the intermixture of two or more ingredients, which develop a different or additional property or properties which the several ingredients individually do not possess in common". On this same page in the Toulmin text appears a quotation from Commercial Acetylene Co. v. Avery Portable Lighting Co. 166 Fed. 907, "There is no restriction as to the nature of the composition which may be patented. The only limitation is they must be new, useful and the result of invention." Thus, it is respectfully submitted that these new *Pseudomonas* bacteria may be considered compositions of matter. On the other hand, in the text Patent Claims by Ridsdale Ellis (Baker, Voorhis & Co., Inc., 1949, page 436) it is pointed out that the authors of the patent statutes intended "manufacture" to mean anything "made" other than those articles which fall within the classes of machines on the one hand and compositions of matter on the other.

It is respectfully submitted that whether the *Pseudomonas* bacteria claimed in Applicant's specification are considered compositions of matter or manufacturers, they clearly are new and different organisms having distinctive capabilities, character and use. In Funk Bros. Seed Co. v. Kalo Inoculant Co., 76 USPQ 280 in the carryover paragraph, page 281 to 282, it is pointed out that "The combination of species produces *no new bacteria, no change* in the six species of bacteria, and *no enlargement of* the range of their *utility*. Each species has the same effect it always had." (emphasis supplied). Thus, presumably, had the patentee produced new, changed bacteria with an enlarged range of utility (as is the case in the instant application), the Court would have been able to conclude that the product claims fully met the statutory requirements.

It is respectfully submitted that the claims 27-32 and 34-36 as amended fully comply with the requirements of 35 U.S.C. 112. Specifically, with respect to claims 27 (couched in Jepson form) in which the term "complex nutrient liquid medium" is recited in the introductory portion thereof, since several such liquid nutrient media may be used, it is respectfully submitted that this aspect of the invention is not critical and, as a result, more generally terminology should be permitted.

With respect to the rejection of claims 1, 4, 18 and 21 under 35 U.S.C. 103 as unpatentable over the Annual Review article, this reference has been examined and, it is respectfully submitted, that none of the plasmids replicated include *energy-generating plasmids* of any sort.

Claim 30 has been rejected under 35 U.S.C. 103 as unpatentable over the Annual Review article. This ground of rejection is respectfully traversed for the reason set forth hereinabove.

Thus, it is respectfully submitted that claims 7-9, 13, 15, 17, 21, 24-32 and 34-36 fully comply with all statutory requirements and patentably define over the reference cited. Allowance of these claims and passage of the case to issue are respectfully requested.

In the event the Examiner in charge of this application desires to initiate a telephone interview, the undersigned may be reached at the following number.

Area Code 518
346-8771, Ext. 6514.

Respectfully submitted,

LEO I. MALOSSI
Leo I. MaLossi, Attorney
Reg. No. 18,990

AMENDMENT, APRIL 8, 1974

Honorable Commissioner of Patents,

SIR:

In response to the Final Rejection dated January 11, 1974, please amend the above-identified application as follows in accordance with the provisions of Rule 116:

Page 30, line 20, delete "source" and insert —source—

Page 42, line 21, delete "(RD-5561)" and insert —260,-488—; after "filed" insert —June 7—

For Claim 30, see Allowed Claim 30.

* * * * *

REMARKS

The Final Rejection of claims 7-9, 13, 15, 17, 21, 24-26 and 30-32 is respectfully traversed. Withdrawal of the grounds of rejection set forth therein is respectfully requested. The allowance of claims 27-29, the indication of the allowability of claims 34-36 and the statement that claims 30-32 are free of the prior art are respectfully noted.

With respect to the rejection of claims 7-9, 13, 15, 17, 21 and 24-26 under 35 U.S.C. 101 as not being within the statutory classes, this ground of rejection is respectfully traversed. It is respectfully submitted that Applicant has presented claims to a patentable composition of matter or, in the alternative to a manufacture, both of which constitute statutory classifications provided for by 35 U.S.C. 101.

As is clearly stated in *Merck & Co., Inc.* v. *Olin Mathieson Chemical Corp.*, 116 USPQ 484, 488 "There is nothing in the language of the Act which precludes the issuance of a patent upon a 'product of nature' when it is a 'new and useful composition of matter' and there is compliance with the specified conditions for patentability. All of the tangible things with which man deals and for

which patent protection is granted are products of nature in the sense that nature provides the basic source materials. The 'matter' of which patentable new and useful compositions are composed necessarily includes naturally existing elements and materials."

The court in *Riter-Conley Mfg. Co.* v. *Aiken et al*, 203 Fed. 699, 703 in quoting from an earlier case, states that "the term 'manufacture' as used in the patent law, has a very comprehensive sense, embracing whatever is made by the art or industry of man, not being a machine, a composition of matter, or a design,". Also, additional discussion of the term "manufacture" on page 701 of this case points out that "From its original derivation of 'facere manu', or handworked products, the word has broadened into all means of treating raw materials . . ." (underlining added).

It is respectfully submitted that in view of such statements by the courts, Applicant's claims fall into one or the other (or both) statutory class of "composition" and "manufacture". It is further respectfully submitted that, since Applicant's invention has been fully, clearly, concisely and exactly described in Applicant's specification, the invention is claimable as a new and unobvious composition or, at the least, as a new and unobvious manufacture. And, further, these claims merit patenting, because no evidence has been produced that the organisms claimed a) existed before, either naturally or by deliberate construction or b) have been specifically suggested in the art together with directions for their preparation.

The accompanying Declaration Under Rule 132 by Dr. Ananda M. Chakrabarty is presented to clarify the nature of the change that occurs in the parent microorganism, when a degradative plasmid has been introduced thereinto. As is pointed out in the Declaration, a cooperative activity ensues between the plasmid and the parent microorganism, once compatibility has been established. As a result, the genetically engineered organism achieves a capability for degrading various crude oil components not present in the parent cell and, thereafter, as replication occurs, all of the genetic information passes on to

future strains. Thus, it is respectfully submitted that a *fundamental* alteration has occurred in the parental cell so that it is erroneous to conclude that the genetically engineered organism is "a thing occurring in nature that is substantially unaltered".

The Supreme Court of the United States in *Funk Bros. Seed Co.* v. *Kalo Inoculant Co.* 333 US 127, 68 SC 440, 76 USPQ 280 sets forth clear and distinct rules by which it may be recognized whether the qualities of the bacterium set forth in a patent claim are merely "the work of nature" or whether some law or nature has actually been applied to a new and useful end (76 USPQ 281, column 2—p282, column 1). Thus, it appears that in order to establish that some law of nature has been applied to a new and useful end, the Court would expect to see that:

a) a new bacterium has been produced.
b) a change in the bacterium has been brought about or
c) an enlargement in the range of the utility of the bacterium has resulted.

It is respectfully submitted that in the instant application, Applicant has adequately met this test having produced genetically engineered organisms, that are very markedly changed from the parent cells, which parent cells after alteration embody (and will reproduce) in a single organism capabilities never found to exist in a single organism before.

Thus, it is respectfully submitted that the guidelines provided by the Supreme Court together with the information provided in the enclosed Declaration much more adequately deal with the questions of the patentability of Applicant's claims than such case law as *Ex parte Grayson* 51 USPQ 413 (shrimp), *American Food Growers, Inc.* v. *Brogdex Company* 8 USPQ 131 (orange) or *Hartranft* v. *Wiegmann* 121 USPQ 609 (shells), none of which relate to bacteria and, more importantly, none of which involve a fundamental alteration of the natural article.

The rejection of claims 30-32 under 35 U.S.C. 112 is respectfully traversed. Claim 30 (and thereby claims 31 and 32) has been amended to specifically recite *Pseudomonas*.

Thus, it is respectfully submitted that claims 7-9, 13, 15, 17, 21, 24-26 and 30-32 (together with allowed and allowable claims 27-29 and 34-36) fully comply with the statutory requirements for patentability. Allowance of all claims and passage of the case to issue are respectfully requested.

In the event the Examiner in charge of this application cannot agree as to the patentability of these claims, entry of this amendment (and Declaration) for purposes of appeal is respectfully requested.

In the event the Examiner in charge of this application desires to initiate a telephone interview, the undersigned may be reached at the following number:

Respectfully submitted,

LEO I. MALOSSI
Leo I. MaLossi, Attorney
Reg. No. 18,990

April 5, 1974

DECLARATION OF CHAKRABARTY

I, Ananda M. Chakrabarty, of the General Electric Company Research and Development Center, Schenectady, New York, declare that:

1. After receiving the degree of Doctor of Philosophy in biochemistry from Calcutta University in 1965, I joined the University of Illinois (Urbana, Illinois) as a Post Doctoral Research Associate. Since leaving the University of Illinois in 1971, I have been employed as a Staff Microbiologist by the General Electric Company at the Research and Development Center (R&DC). Both during my post doctoral work and while on the staff at the R&DC, I have been engaged in investigations in viral transmission of genetic materials in microorganisms and in the construction of genetically engineered microorganisms for the production of useful materials.

2. In connection with my research efforts, I have been the author or co-author of approximately 25 technical papers in the above areas of investigation.

3. I am the inventor in the above-identified application and wish to comment on the extent to which the *Pseudomonas* bacterium claimed (hereinafter referred to as "genetically engineered organism" or "GEO") therein is different from the parental cell organism (PC).

The GEO has been constructed as a genetically improved strain by transferring to the PC genetic materials from other microorganism(s), each of which has the capability for degrading a different component of crude oil. The genetic materials are transferred in the form of plasmids, which become physically incorporated into the cellular structure of the PC. The genetic materials so introduced are either naturally compatible with any other plasmid in the PC or are deliberately rendered compatible by fusion. Thereafter, these genetic materials become stably inheritable.

Once stabilized, not only is the plasmid-supplied genetic material tolerated by the PC, but a mutual cooperation results. Thus, at the direction of the plasmid, the PC operates its protein-generating machinery to produce

hydrocarbon-degrading enzymes, which are completely new within the cellular environment of the PC. This provides to the PC a new capability for degrading one or more crude oil components depending upon the number of plasmids transferred. Thereafter, as the PC replicates, all this genetic information passes on to the daughter strains for an indefinite number of generations.

Thus, I have not simply brought about a mutational change of the genetic material but have brought to the PC substantial quantities of new genetic material (as much as 25% more DNA) from different microorganisms resulting in altered composition of the cellular material of the PC. The fact that this is a *fundamental* alteration is evidenced by the relative survival capabilities of the GEO and the PC in crude oil and in Bunker C. These growth capabilities are described in the specification of the above-identified application (page 29, line 17—page 34, line 8) and in the drawing thereof.

The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

April 5, 1974
Date

ANANDA M. CHAKRABARTY
Ananda M. Chakrabarty

REJECTED CLAIMS

7. A bacterium from the genus *Pseudomonas* containing therein at least two stable energy-generating plasmids, each of said plasmids providing a separate hydrocarbon degradative pathway.

8. The *Pseudomonas* bacterium of claim 7, said bacterium being of the specie *P. aeruginosa.*

9. The Pseudomonas bacterium of claim 7, said bacterium being of the specie *P. putida.*

13. The *Pseudomonas* bacterium of claim 7 wherein the hydrocarbon degradative pathways are selected from the group consisting of linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic.

15. The *P. aeruginosa* bacterium of claim 8 wherein the bacterium contains CAM, OCT, SAL, and NPL plasmids.

17. The *P. putida* bacterium of claim 9 wherein the bacterium contains CAM, SAL, NPL and RP-1 plasmids.

21. An inoculum for the degradation of a pre-selected substrate comprising a complex or mixture of hydrocarbons, said inoculum consisting essentially of bacteria of the genus *Pseudomonas* at least some of which contain at least two stable energy-generating plasmids, each of said plasmids providing a separate hydrocarbon degradative pathway.

24. The inoculum of claim 21 wherein the hydrocarbon degradative pathways are selected from the group consisting of linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic.

25. The inoculum of claim 24 wherein the bacteria having multiple energy-generating plasmids are of the specie *P. aeruginosa.*

26. The inoculum of claim 24 wherein the bacteria having multiple energy-generating plasmids are of the specie *P. putida.*

ALLOWED CLAIMS

27. In the process in which a first energy-generating plasmid specifying a degradative pathway is transferred by conjugation from a donor *Pseudomonas* bacterium to a recipient *Pseudomonas* bacterium containing at least one energy-generating plasmid that is incompatible with said

first plasmid, said transfer ocurring in the quiescent state after the mixing of substantially equal volumes of cultures of said donor and said recipient, each culture presenting the respective organisms in a complex nutrient liquid medium at a population density of at least about 1,000,000 cells/ml, the improvement wherein after conjugation has occurred, the multi-plasmid conjugatant bacteria are subjected to DNA-cleaving radiation in a dosage sufficient to fuse the first plasmid and the plasmid incompatible therewith located in the same cell.

28. The improvement of claim 27 wherein the DNA-cleaving radiation is UV radiation.

29. The improvement of claim 28 wherein the first plasmid provides the degradative pathway for camphor and the recipient *Pseudomonas* contains the degradative pathway for n-octane.

30. An inoculated medium for the degradation of liquid hydrocarbon substrate material floating on water, said inoculated medium comprising a carrier material able to float on water and bacteria from the genus *Pseudomonas* carried thereby, at least some of said bacteria each containing at least two stable energy-generating plasmids, each of said plasmids providing a separate hydrocarbon degradative pathway and said carrier material being able to absorb said hydrocarbon material.

31. The inoculated medium of claim 30 wherein the carrier material is straw.

32. The inoculated medium of claim 30 wherein the hydrocarbon degradative pathways are selected from the group consisting of linear aliphatic, cyclic aliphatic, aromatic and polynuclear aromatic.

35. The inoculated medium of claim 30 wherein the bacteria are of the specie *P. aeruginosa.*

36. The inoculated medium of claim 30 wherein the bacteria are of the specie *P. putida.*

Filed
U.S. Court of Customs
and Patent Appeals
Dec 28 '76
George E. Hutchinson
Clerk

PETITION OF APPEAL

To the United States Court of Customs
and Patent Appeals:

Your Petitioner, Ananda M. Chakrabarty, of Latham, New York, respectfully represents:

That he is the original and first inventor of a certain new and useful bacterium from the genus *Pseudomonas.*

That on June 7, 1972, in the manner prescribed by law, he presented his application to the Patent and Trademark Office praying that a patent be issued to him for said invention.

That proceedings were had in said Office upon said application; that on May 20, 1976, and October 19, 1976, said application was rejected by the Board of Appeals and a patent for said invention as set forth in claims 7, 8, 9, 13, 15, 17, 21, 24, 25 and 26 of said application was refused him.

That on November 18, 1976, your Petitioner pursuant to Section 142 of Title 35, United States Code, gave notice to the Commissioner of Patents of his appeal to this Honorable Court from his refusal to issue a patent to him for the invention set forth in said claims and filing with him in writing the special reasons of appeal.

That the Commissioner of Patents has furnished him a certified transcript of the record and proceedings relating to said application for patent including the Notice and Reasons of Appeal, which transcript is filed herewith together with a check for fifty dollars ($50.00) for the filing fee.

Wherefore, your Petitioner prays that his appeal may be heard upon and, for the reasons assigned therefor to

the Commissioner, that the decision of the Commissioner be reversed.

Respectfully submitted,

ANANDA M. CHAKRABARTY,
By his attorney,
Leo I. MaLossi JBF
LEO I. MALOSSI
P.O. Box 8
Schenectady, New York 12309

December 27, 1976

Of Counsel

Joseph B. Forman
JOSEPH B. FORMAN
2001 Jefferson Davis Highway
Arlington, Virginia 22202

UNITED STATES COURT OF CUSTOMS AND PATENT APPEALS

Appeal No. 77-535
Serial No. 260,563

IN THE MATTER OF THE APPLICATION OF
ANANDA M. CHAKRABARTY

ORDER

The Supreme Court of the United States having vacated the judgment in *Parker* v. *Bergy, et al.* on June 22, 1978, and remanded that case for further consideration in light of *Parker* v. *Flook*, decided by the Supreme Court on June 22, 1978; and the Commissioner of Patents and Trademarks having petitioned to vacate the judgment and recall the mandate in this appeal in view of that action of the Supreme Court;

IT IS ORDERED

That the petition is granted to the extent that the judgment in this appeal is vacated, the mandate in this appeal is recalled and this appeal is restored to the calendar.

IT IS FURTHER ORDERED

That the parties shall file supplementary briefs directed solely to the effect of *Parker* v. *Flook* on this appeal. Briefs shall be filed in accordance with the following schedule:

Appellant's Brief—40 days after date of this order
Commissioner's Brief—70 days after date of this order
Reply Brief—84 days after date of this order

The case will be set for hearing on November 6, 1978.

FOR THE COURT

/s/ Howard T. Markey
HOWARD T. MARKEY
Chief Judge

Dated: 11 Aug. 78

SUPREME COURT OF THE UNITED STATES

No. 79-136

LUTRELLE F. PARKER, ACTING COMMISSIONER OF PATENTS AND TRADEMARKS, PETITIONER

v.

MALCOLM E. BERGY, ET AL,; and

LUTRELLE F. PARKER, ACTING COMMISSIONER OF PATENTS AND TRADEMARKS, PETITIONER

v.

ANANDA M. CHAKRABARTY

ORDER ALLOWING CERTIORARI

Filed October 29, 1979

The petition herein for a writ of certiorari to the United States Court of Customs and Patent Appeals is granted.

☆ U. S. GOVERNMENT PRINTING OFFICE: 1979 305233 122